ANTI-ANGIOGENIC AND VASCULOPROTECTIVE EFFECT OF *PUNICA GRANATUM* ROOT

Dissertation Submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,

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In partial fulfilment for the requirements for the award of the degree of

MASTER OF PHARMACY

IN

BRANCH – IV- PHARMACOLOGY

Submitted by

A.SUBASH CHANDRA BOSE

REGISTER NO: 261525505

Under the guidance of

Dr. V. Rajesh, M. Pharm., Ph.D.,

Professor and Head,

Department of Pharmacology,

THE ERODE COLLEGE OF PHARMACY AND RESEARCH INSTITUTE,

ERODE- 638112.

OCTOBER - 2017

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"Anti-angiogenic and vasculoprotective effect of *Punica granatum* root"** submitted by **Register No. 261525505** to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in partial fulfilment for the degree of **Master of Pharmacy** in **Pharmacology** is the bonafide work carried out under guidance and direct supervision of **Dr. V. Rajesh, M.Pharm., Ph.D.,** Professor and Head, Department of Pharmacology, **The Erode College of Pharmacy and Research Institute**, **Erode-638112** and was evaluated by us during the academic year 2016-2017.

1. INTERNAL EXAMINER **2. EXTERNAL EXAMINER**

3. CONVENER OF EXAMINATION

Examination Centre: The Erode College of Pharmacy and Research Institute.

Date:

The Erode College of Pharmacy and Research Institute

Dr. V. Ganesan, M.Pharm., Ph.D.,

Principal,

Professor and Head, Department of Pharmaceutics,

The Erode College of Pharmacy and Research Institute,

Erode - 638112.

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Place: Erode

Date:

Dr. V. Ganesan, M.Pharm., Ph.D.,

Principal

The Erode College of Pharmacy and Research Institute

Dr. V. Rajesh, M.Pharm., Ph.D.,
Professor and Head,
Department of Pharmacology,
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PLACE: ERODE
DATE:

Dr. V. Rajesh, M.Pharm., Ph.D.,
Professor and Head

DECLARATION

I do hereby declare that the dissertation work entitled **"Anti-angiogenic and vasculoprotective effect of *Punica granatum* root"** submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in the partial fulfilment for the Degree of **master of pharmacy in pharmacology**, was carried out by myself under the guidance and direct supervision of **Dr. V.Rajesh,M.Pharm., Ph.D.,** Professor and Head, Department of Pharmacology**, The Erode College of Pharmacy and Research Institute, Erode-638112,** during the academic year 2016-2017.

This work is original and has not been submitted in part or full for the award of any other Degree or Diploma of this or any other University.

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Date

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ABBREVIATIONS

Mmp3	Metalloproteinase 3
VEGF	Vascular endothelial growth factor
bFGF	Basic fibroblast growth factor
CDI	Cartilage-derived inhibitor
hCG	Human chorionic gonadotropin
PRP	Proliferin-related protein
IP-10	Interferon inducible protein-10
TIMP-1	Tissue inhibitor of metalloproteinase-1
TKIs	Tyrosine kinase inhibitors
mTOR	Mammalian target of rapamycin).
mCRC	Metastatic colorectal cancer
NSCLC	Non-small cell lung cancer
RCC	Metastatic renal cell cancer
DTC	Differentiated thyroid carcinoma
GIST	Gastrointestinal stromal tumor
SEGA	Subependymal giant cell astrocytoma
PNET	Progressive neuroendocrine tumors
DR	Diabetic Retinopathy
IDDM	Insulin dependent diabetic mellitus

NIDDM	Non-Insulin dependent diabetic mellitus
MA	Microaneurysms
NPDR	Mild Nonproliferative Diabetic Retinopathy
PDR	Proliferative Diabetic Retinopathy
CAM	Chick chorioallantoic membrane
LDL	Low-density lipoprotein
PFE	Pomegranate fruit extract
COPD	Chronic obstructive pulmonary disorder
CHF	Congestive heart failure
ED	Erectile dysfunction
CEE	Crude ethanolic extract
QRT-PCR	Quantitative reverse transcriptase–polymerase chain reaction
TG	Triglyceride
PPE	Pomegranate peel extract
HUVECs	Human umbilical resin endothelial cells
GAE	Gallic acid equivalents
IC_{50}	Inhibiting concentration
CE	Crude ethanol Extract
PEF	Petroleum Ether Fraction
CF	Chloroform Fraction

EAF	Ethyl Acetate Fraction
BF	n-butanol fraction
AF	Aqueous Fraction
SEM	Standard error of the mean
ANOVA	One-way analysis of variance
EEPG	Ethanol extract of *Punica granatum*
TPC	Total Phenolic content
TFC	Total Flavonoid content
TAC	Total antioxidant capacity
TTC	Total tannin content
Cme-ot	Crude ethanol extract

CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Angiogenesis

Formation of new blood vessels - might one day be manipulated to treat disorders from cancer to heart disease. Blood vessels play surprisingly central roles in many serious chronic disorders. Blood vessels constitute the first organ in the embryo and form the largest network in our body but, sadly, are also often deadly. When dysregulated, the formation of new blood vessels contributes to numerous malignant, ischemic, inflammatory, infectious and immune disorders.

In mammalian development, a vascular network is formed throughout the body, except in avascular tissues (e.g., cornea and intervertebral disks), to meet the tissue requirements for oxygen and nutrients. Three major processes are necessary to form a complete vascular network- vasculogenesis, angiogenesis and vascular remodeling.

Vasculogenesis denotes *de novo* blood vessel formation, in which vascular precursor cells (angioblasts) migrate to sites of vascularization, differentiate into endothelial cells and coalesce to form the initial vascular plexus.

Angiogenesis refers to the budding of new capillary branches from existing blood vessels. Vascular remodeling describes a later phase when a newly formed vessel increases its luminal diameter in response to increased blood flow and acquires identity as an artery, vein or capillary.

Once these three processes are completed during postnatal development, adult vasculature is stable and rarely proliferates under physiological conditions. However, in pathological situations such as ocular neovascular diseases and cancer, existing vessels again start to grow to meet the abnormal requirements for oxygen and nutrients of the pathologically expanding tissues.

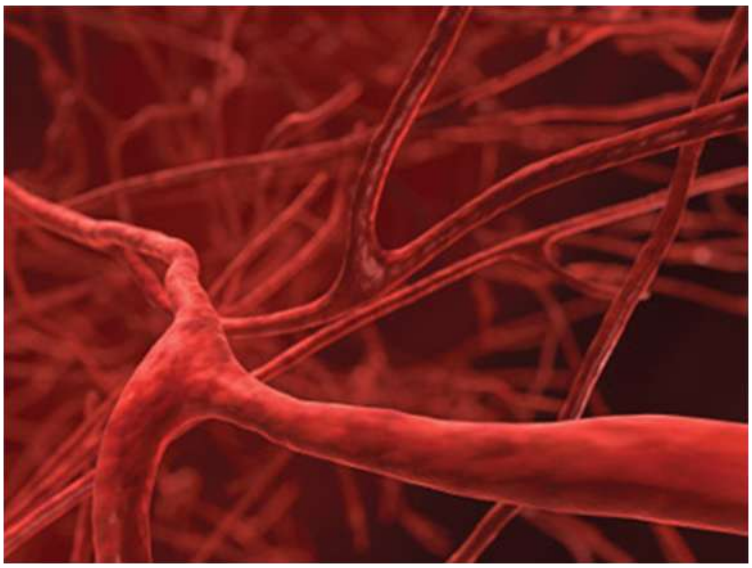


Figure.1 Blood vessels

The angiogenesis process

Angiogenesis occurs as an orderly cascade of molecular and cellular events

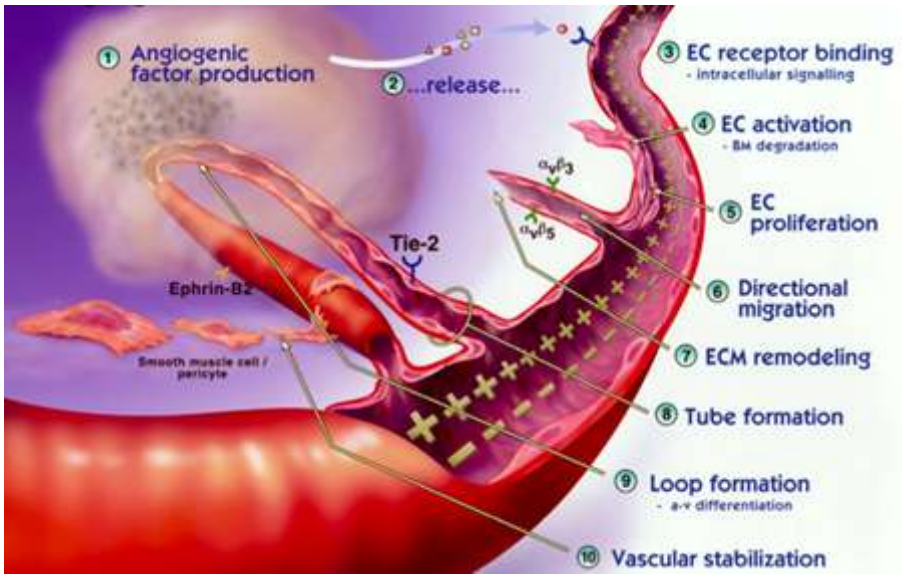


Figure.2 Angiogenesis- cascade of events

Cascade of events in angiogenesis

1. Angiogenic growth factors bind to their receptors on the surface of endothelial cells in pre-existing venules (parent vessels).

2. Growth factor-receptor binding activates signalling pathways within endothelial cells.
3. Activated endothelial cells release proteolytic enzymes that dissolve the basement membrane surrounding parent vessels.
4. Endothelial cells proliferate and sprout outward through the basement membrane.
5. Endothelial cells migrate into the wound bed using cell surface adhesion molecules known as integrins (αVβ3, αVβ5, and α5β1).
6. At the advancing front of sprouting vessels, enzymes known as matrix metalloproteinases (MMPs) dissolve the surrounding tissue matrix.
7. Vascular sprouts form tubular channels which connect to form vascular loops.
8. Vascular loops differentiate into afferent (arterial) and efferent (venous) limbs.
9. New blood vessels mature by recruiting mural cells (smooth muscle cells and pericytes) to stabilize the vascular architecture.
10. Blood flow begins in the mature stable vessel.

These complex growth factor-receptor, cell-cell, and cell-matrix interactions characterize the angiogenesis process, regardless of the inciting stimuli or its location in the body.

Angiogenesis in disease

In many serious disease states, angiogenesis is dysregulated. Angiogenesis-dependent diseases result when new blood vessels either grow excessively or insufficiently.

Figure.3 Angiogenesis in disease

Excessive angiogenesis

- Occurs in diseases such as cancer, diabetic blindness, age-related macular degeneration, rheumatoid arthritis, psoriasis, and more than 70 other conditions.
- Occurs when diseased cells and tissue produce abnormal amounts of angiogenic growth factors, overwhelming the effects of natural angiogenesis inhibitors.
- In these conditions, new blood vessels grow and feed diseased tissues; the new vessels are abnormal and leaky and can destroy normal tissues. In the case of cancer, the abnormal vessels allow tumor cells to escape into the circulation and lodge in other organs (tumor metastasis).
- Antiangiogenic therapies, aimed at halting new blood vessel growth, are used to treat these conditions.

Insufficient angiogenesis

- Occurs in diseases such as coronary artery disease, stroke, and chronic wounds.
- In these conditions, blood vessel growth is inadequate, and circulation is not properly restored, leading to the risk of tissue death.
- Insufficient angiogenesis occurs when tissues do not produce adequate amounts of angiogenic growth factors.
- Therapeutic angiogenesis therapies, aimed at stimulating new blood vessel growth with growth factors, are being developed to treat these conditions.

Table.1 Abnormal angiogenesis/vascular malfunction (Carmeliet and Jain, 2000)

Organ	Processess characterized by abnormal angiogenesis/vascular malfunction
Blood vessels	Atherosclerosis, Haemangioma, Heamangioendothelioma (increased vascularisation) Vascular malformations (Abnormal remodelling)
Skin	Warts, Pyogenic granulomas, Hair growth, Kaposis sarcoma, scar keloids, allergic oedema, neoplasms (Increased vascularisation). Psoriasis (Skin vessels enlarge and become tortuous) (Abnormal remodelling). Decubitus or Stasis ulcers, Gastrointestinal ulcers. (Insufficient vascularisation)
Uterus, Ovary, Placenta	Dysfunctional uterine bleeding, Follicular cysts, Ovarian hyperstimulation, endometriosis, neoplasms (increased vascularisation). Pre-eclampsia (Abnormal remodelling). Placental insufficiency (Insufficient vascularisation).
Peritoneum, Pleura	Respiratory distress, Ascites, Peritoneal sclerosis (dialysis patients), adhesion formation (abdominal surgery), metastatic spreading (Increased vascularisation and/permeability).
Heart, Skeletal muscle	Work overload (increased vascularisation) Ischemic heart and limb disease (Insufficient vascularisation).
Adipose tissue	Obesity (increased vascularisation)
Bone, Joints	Rheumatoid arthritis, Synovitis, Bone and cartilage destruction, Osteomyelitis, Pannus growth, Osteophyte formation, Cancer (increased vascularisation). Aseptic necrosis, impaired healing of fractures (Insufficient vascularisation).
Liver, Kidney, ear, lung and other epithelia	Inflammatory and infectious processes (Hepatitis, Pneumonia, Glomerulonephritis), asthma, nasal polyps, transplantation, liver regeneration, cancer (increased vascularisation). Pulmonary hypertension, diabetes (Abnormal remodelling). Pulmonary and systemic hypertension (Insufficient vascularisation).
Brain, nerves and eye	Retinopathy and prematurity, diabetic retinopathy, choroidal and other intraocular disorders, leukomalacia, cancer (increased vascularisation). Stroke, Vascular dementia, Alzheimers disease, CADASIL (Insufficient vascularisation).
Endocrine organs	Thyroditis, Thyroid enlargement, Pancreas transplantation (increased vascularisation). Thyroid pseudocyst (Insufficient vascularisation).
Haematopoiesis	AIDS, Haematologic malignancies (increased vascularisation).

Angiogenesis in diseases

Angiogenesis and diabetic retinopathy

It is evident that the complication of diabetes is characterized by the formation of new vessels inside the retina showing abnormal architecture and permeability. The pathogenesis behind visual loss is related with retinal angiogenesis and increased retinal vascular permeability. Several mechanisms have been proposed to cause the retinal and vasculature cellular damage. High blood glucose induces hypoxia in retinal tissues, thus leading to the production of VEGF-A (vascular endothelial growth factor protein). Hypoxia is a key regulator of VEGF induced ocular neovascularisation.

A persistent increase in blood glucose levels shunts excess glucose into the aldose reductase pathway in certain tissues, which converts sugars into alcohol (e.g., glucose into sorbitol, galactose to dulcitol). Intramural pericytes of retinal capillaries seem to be affected by this increased level of sorbitol, eventually leading to the loss of its primary function (i.e, autoregulation of retinal capillaries) (Crawford et al. 2009).

Angiogenesis and Cancer

Like tissues of the body, tumors need a blood supply. They get this through new blood vessel formation that extends into the tumour (angiogenesis). The nutrients and growth factors supplied by these blood vessels allow tumors to grow.

Before the 1960s, cancer researchers believed that the blood supply reached tumors simply because pre-existing blood vessels dilated. But later experiments showed that angiogenesis - the growth of the new blood vessels is necessary for cancerous tumors to keep growing and spreading.

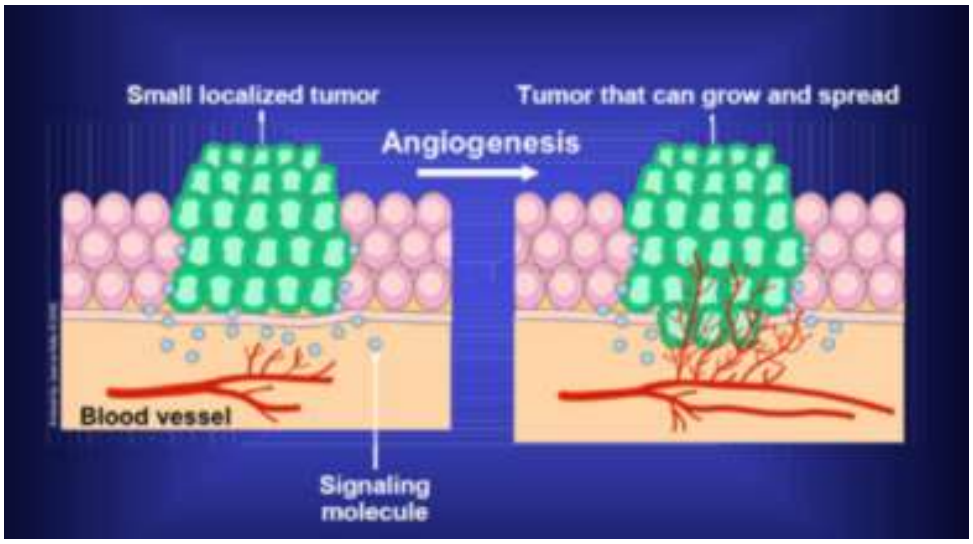


Figure.4 Tumour angiogenesis

Cancer researchers became interested in angiogenesis factors in 1968, when the first hints emerged that tumour might release such substance to foster their own progression. Two independent research teams- Melvin Greenblatt of the University of Southern California, working with Philipe Shubik of the University of Chicago, and Robert L Eharmann and Mogens Knoth of Harvard Medical School showed that burgeoning tumours, release unidentified substance that induces existing blood vessels to grow into them. Such proliferation promotes tumour growth because it ensures the rich supply of blood loaded with oxygen and nutrients. In 1971, Judah Folkman of Harvard proposed that interfering with this factor might be a way to kill tumours, by starving them of a blood supply. Folkman later posited that blocking the factor would slow cancer's spread, a process called metastasis, because cancer cells must enter blood vessels to travel to other part of the body.

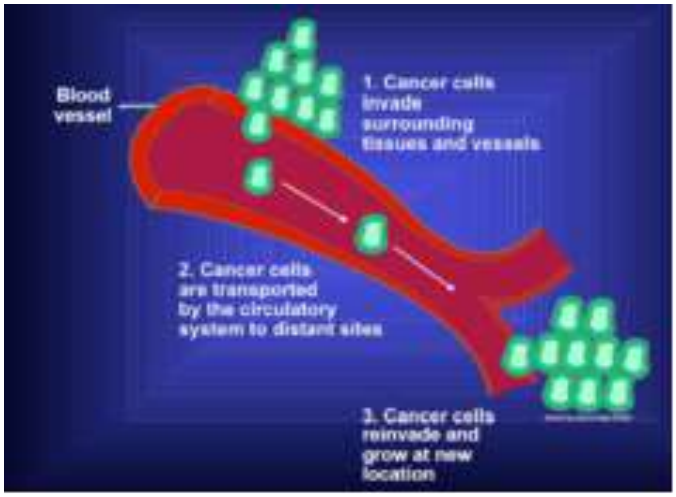


Figure.5 Metastasis

Tumour Angiogenesis

Tumour angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. Tumour angiogenesis actually starts with cancerous tumour cells releasing molecules that send signals to surrounding normal host tissue. This signal activates certain genes in the host tissue that, in turn, make proteins to encourage growth of new blood vessels. For the tumour to grow beyond 2-3 mm in size, neovascularization is required. This finding paved the way for new anti-angiogenic cancer therapies aimed at disrupting angiogenesis signalling pathways (http://cancer.gov/cancertopics/understandingcancer).

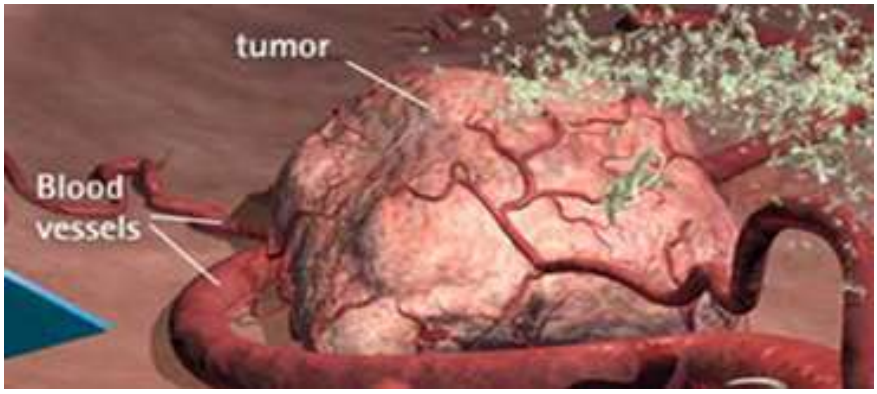


Figure.6 Large highly vascularised tumour

Activators of Angiogenesis

Once researchers knew that cancer cells could release molecules to activate the process of angiogenesis, the challenge became to find and study these angiogenesis-stimulating molecules in animal and human tumors. From such studies more than a dozen different proteins, as well as several smaller molecules, have been identified as "angiogenic," meaning that they are released by tumors as signals for angiogenesis. Many of them are growth factors that induce endothelial cells to divide, migrate directionally toward the inducing stimulus, and differentiate into tubular structures. Among these molecules, two proteins appear to be the most important for sustaining tumor growth: vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). VEGF and bFGF are produced by many kinds of cancer cells and by certain types of normal cells, too. (http://cancer.gov/cancertopics/understandingcancer).

There are at least 20 different known angiogenic growth factors.

Naturally occurring activators of angiogenesis

Proteins

- Acidic fibroblast growth factor
- Angiogenin
- Angiopoietin-1
- Follistatin
- Basic fibroblast growth factor (bFGF)
- Epidermal growth factor
- Granulocyte colony-stimulating factor
- Hepatocyte growth factor
- Interleukin 8
- Leptin

- Midkine
- Placental growth factor
- Platelet-derived endothelial growth factor
- Progranulin
- Proliferin
- Platelet-derived growth factor-BB
- Pleiotrophin
- Scatter factor/ Hepatocyte grouth factor
- Transforming growth factor alpha
- Transforming growth factor-beta
- Tumour necrosis factor alpha
- Vacular endothelial growth factor (VEGF)

Small molecules

- Adenosine
- 1-Butyryl glycerol
- Nicotinamide
- Prostaglandins E_1 and E_2 (Polverini, 1995; Prabhu et al., 2011)

Angiogenesis signalling Cascade

Mediators can stimulate angiogenesis directly by interacting with receptors on the endothelial cell surface, or indirectly by attracting and activating accessory cells, *i.e.*, inflammatory macrophages, and inducing them to produce angiogenic mediators (Polverini and Leibovich, 1984). Others, such as copper, may function as co-factors in key interstitial enzyme systems or, in the case of plasminogen activator, can activate latent enzymes such as transforming growth factor β to reveal its angiogenic (or angiostatic) activity (Roberts and Sporn, 1989). Still others play a key role in stabilizing and/or enhancing the function of stimulatory molecules normally sequestered in the extracellular matrix surrounding blood

vessels, as heparin does, which when bound to basic fibroblast growth factor facilitates its interaction with high-affinity receptors on the endothelial cell surface (Folkman and Klagsbrun, 1987; Vlodavski *et al.*, 1987).

VEGF and bFGF are first synthesized inside tumor cells and then secreted into the surrounding tissue. When they encounter endothelial cells, they bind to specific proteins, called receptors, sitting on the outer surface of the cells. The binding of either VEGF or bFGF to its appropriate receptor activates a series of relay proteins that transmits a signal into the nucleus of the endothelial cells. The nuclear signal ultimately prompts a group of genes to make products needed for new endothelial cell growth.

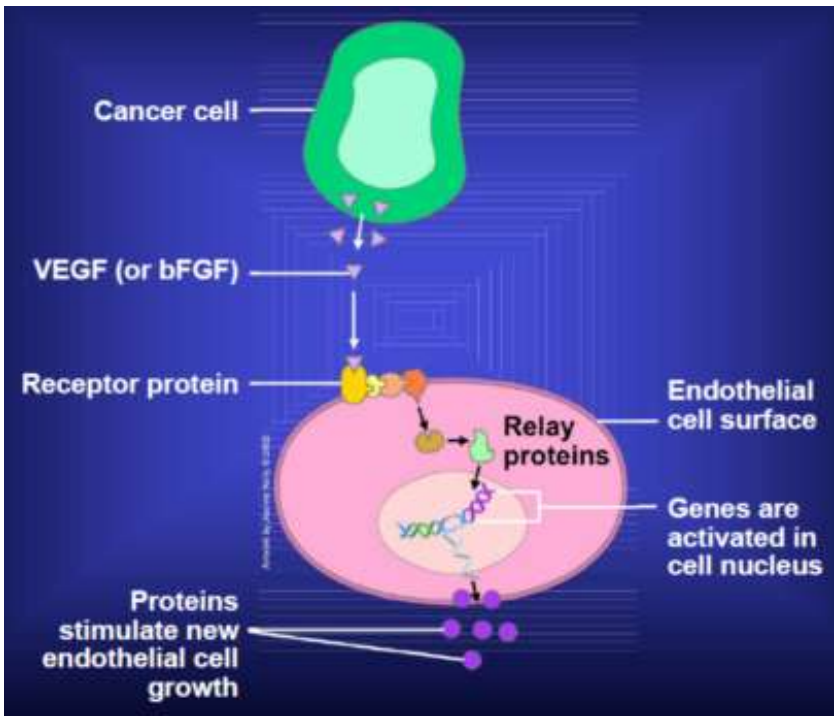


Figure.7 Angiogenesis signaling cascade

Endothelial Cell Activation

The activation of endothelial cells by VEGF or bFGF sets in motion a series of steps toward the creation of new blood vessels. First, the activated endothelial cells produce matrix metalloproteinases (MMPs), a special class of degradative enzymes. These enzymes are then

released from the endothelial cells into the surrounding tissue. The MMPs break down the extracellular matrix support material that fills the spaces between cells which is made of proteins and polysaccharides. Breakdown of this matrix permits the migration of endothelial cells. As they migrate into the surrounding tissues, activated endothelial cells begin to divide. Soon they organize into hollow tubes that evolve gradually into a mature network of blood vessels (http://cancer.gov/cancertopics/understandingcancer).

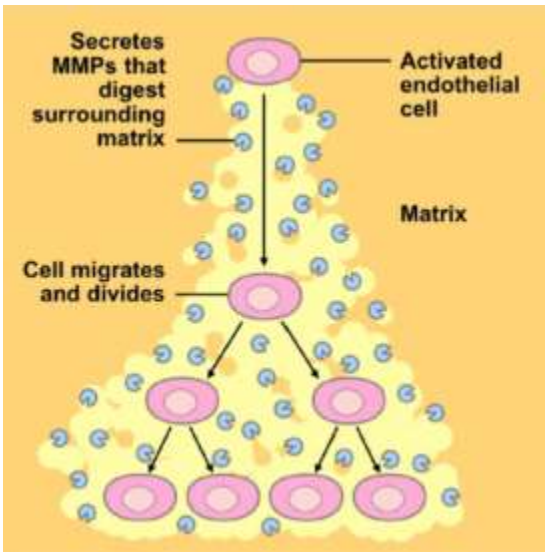


Figure.8 Endothelial cell activation

Inhibitors of Angiogenesis

While the mediators responsible for inducing new capillary growth have been the subject of extensive investigation, only recently attention has focused on the mechanisms and mediators responsible for the timely down-regulation of angiogenesis (Folkman and Klagsbrun, 1987)

Although many tumors produce angiogenic molecules such as VEGF and bFGF, their presence is not enough to begin blood vessel growth. For angiogenesis to begin, these activator molecules must overcome a variety of angiogenesis inhibitors that normally restrain blood vessel growth. Almost a dozen naturally occurring proteins can inhibit angiogenesis.

Among this group of molecules, proteins called angiostatin, endostatin, and thrombospondin appear to be especially important. A finely tuned balance between the concentration of angiogenesis inhibitors and of activators such as VEGF and bFGF determines whether a tumor can induce the growth of new blood vessels. To trigger angiogenesis, the production of activators must increase as the production of inhibitors decreases.

Angiogenesis inhibitors

There are at least 28 known natural angiogenesis inhibitors found in the body (Polverini, 1995; Prabhu et al., 2011)

Proteins

- Angiostatin
- Angioarrestin
- Endostatin
- Antiangiogenic antithrombin III
- Cartilage-derived inhibitor (CDI)
- CD59 complement fragment
- Fibronectin fragment
- Gro-beta
- Heparinases
- Heparin hexasaccharide fragment
- Human chorionic gonadotropin (hCG)
- Interleukin-12
- Kringle 5 (plasminogen fragment)
- 2-Methoxyestradiol
- Placental ribonuclease inhibitor
- Plasminogen activator inhibitor
- Proliferin-related protein (PRP)

- Retinoids
- Tetrahydrocortisol-S
- Transforming growth factor-beta (TGF-b)
- Vasculostatin
- Vasostatin (Calreticulin fragment)
- Interferons alpha/ beta/ gamma
- Interferon inducible protein (IP-10)
- Platelet factor 4
- Prolactin 16Kd fragment
- Thrombospondin
- Tissue inhibitor of metalloproteinase-1 (TIMP-1)
- Tissue inhibitor of metalloproteinase-2 (TIMP-2)
- Tissue inhibitor of metalloproteinase-3 (TIMP-3)

Angiogenesis inhibitors in treatment of cancer

Almost two dozen angiogenesis inhibitors are currently being tested in cancer patients. These inhibitors fall into several different categories, depending on their mechanism of action. Some inhibit endothelial cells directly, while others inhibit the angiogenesis signaling cascade or block the ability of endothelial cells to break down the extracellular matrix.

All cancerous tumour release angiogenic growth factor proteins that stimulate blood vessels to grow into the tumour, providing it with oxygen and nutrients, Anti-angiogenic therapies literally starve the tumour of its blood supply by interfering with this process. New classes of cancer treatments that block angiogenesis are now approved and available to treat cancers of the colon, kidney, lung, breast and liver, as well as multiple myeloma and bone gastrointestinal stromal tumours. Some older drugs have been rediscovered to block angiogenesis

as well. These are being used to treatment angiogenesis dependent conditions, such as hemangiomas, colon polyps and precancerous skin lesions.

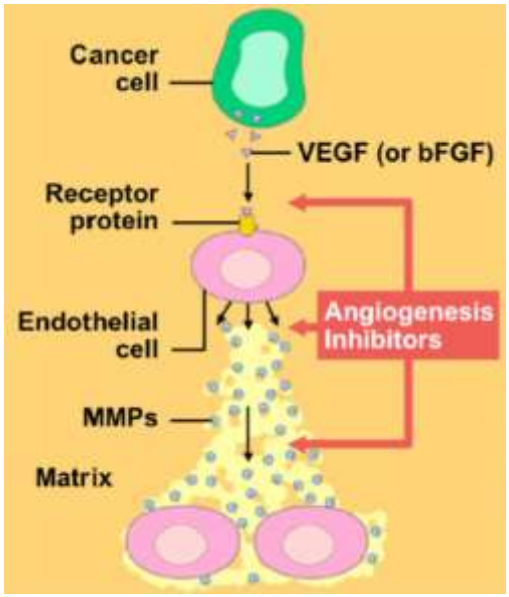


Figure.9 Block the ability of endothelial cells to break down the extracellular matrix

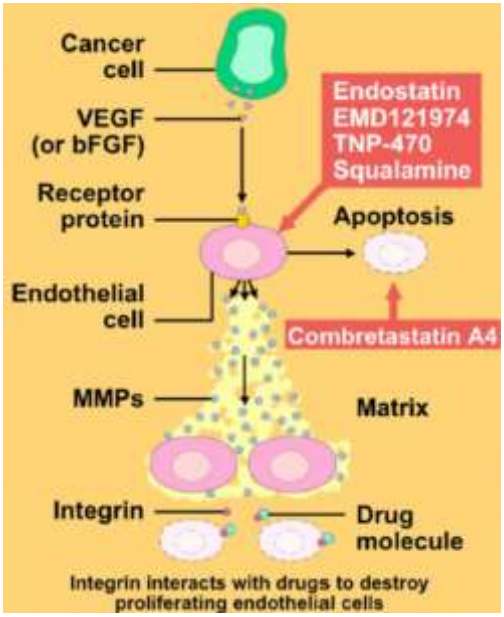


Figure.10 Molecules that directly inhibit the growth of endothelial cells

- Endostatin, the naturally occurring protein known to inhibit tumor growth
- Combretastatin A4 causes growing endothelial cells to commit suicide (apoptosis).
- Integrin promote the destruction of proliferating endothelial cells.

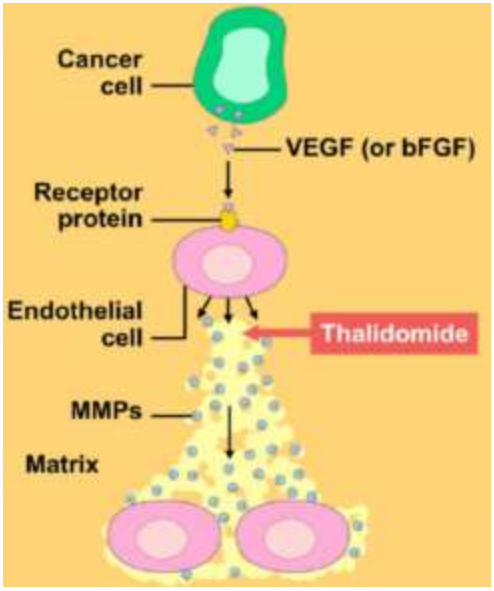


Figure.11 Old drug with a new use

Thalidomide, a sedative used in the 1950s that was subsequently taken off the market because it caused birth defects when taken by pregnant women. Although this drug clearly would not be suitable for pregnant women, its ability to prevent endothelial cells from forming new blood vessels might make it useful in treating non pregnant cancer patients.

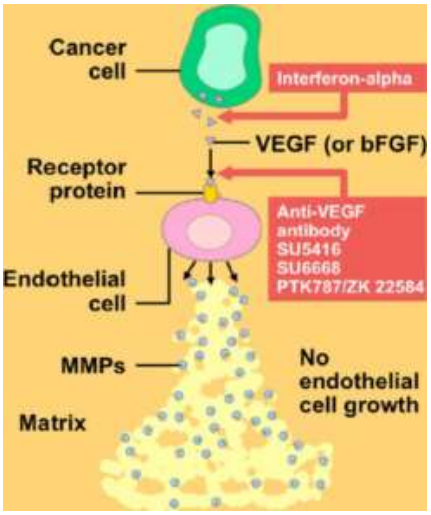


Figure.12 Molecules that interfere with steps in the angiogenesis signaling cascade

- Anti-VEGF antibodies that block the VEGF receptor from binding growth factor.
- Iterferon- alpha, is a naturally occurring protein that inhibits the production of bFGF and VEGF, preventing these growth factors from starting the signaling cascade.
- Several synthetic drugs capable of interfering with endothelial cell receptors.

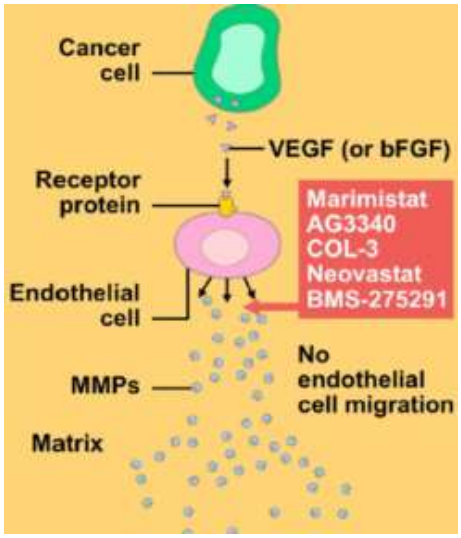


Figure.13 Drugs That Block Extracellular Matrix Breakdown

MMPs are the enzymes that catalyze the breakdown of the extracellular matrix. Because breakdown of the matrix is required before endothelial cells can migrate into surrounding tissues and proliferate into new blood vessels, drugs that target MMPs also can inhibit angiogenesis. Several synthetic and naturally occurring molecules that inhibit the activity of MMPs are currently being tested to see if interfering with this stage of angiogenesis will prolong the survival of cancer patients (http://cancer.gov/cancertopics/understandingcancer).

Approved anti-cancer therapies with recognized antiangiogenic properties

In the U.S., there are currently thirteen approved anti-cancer therapies with recognized antiangiogenic properties in oncology. These agents, which interrupt critical cell signaling pathways involved in tumor angiogenesis and growth, comprise three primary

categories

1) Monoclonal antibodies directed against specific proangiogenic growth factors and/or their receptors.

2) Small molecule tyrosine kinase inhibitors (TKIs) of multiple proangiogenic growth factor receptors.

3) Inhibitors of mTOR (mammalian target of rapamycin).

In addition, there are two other approved anti angiogenic agents may indirectly inhibit angiogenesis through mechanisms that are not completely understood. Angiogenesis inhibitors have also been discovered from natural sources, including tree bark, fungi, shark muscle and cartilage, sea coral, green tea and herbs (liquorices, ginseng, cumin, garlic). In total, more than 300 angiogenesis inhibitors have been discovered to date. Near about 184 million patients in World could benefit from some form of anti angiogenic therapy (Prabhu et al., 2011).

Monoclonal Antibody Therapy

Bevacizumab (Avastin)

A humanized monoclonal antibody that binds biologically active forms of vascular endothelial growth factor (VEGF) and prevents its interaction with VEGF receptors (VEGFR-1 and VEGFR-2), thereby inhibiting endothelial cell proliferation and angiogenesis.

Indications

Metastatic colorectal cancer (mCRC), non-small cell lung cancer (NSCLC), advanced breast cancer, glioblastoma, metastatic renal cell cancer (RCC), advanced ovarian cancer.

- In combination with 5-FU-based chemotherapy as first-line and second-line treatment of mCRC.
- In combination with carboplatin and paclitaxel as first-line treatment of patients with unresectable, locally advanced, recurrent or metastatic non-squamous NSCLC.

- In combination with paclitaxel as first-line treatment in patients with locally recurrent or metastatic breast cancer.
- Second-line treatment of patients with glioblastoma following temozolomide failure.
- In combination with interferon-alfa as first-line treatment of RCC.
- In combination with chemotherapy as first-line treatment in patients with newly diagnosed, advanced ovarian cancer.

Small Molecule Tyrosine Kinase Inhibitors (TKIs)

Seven TKIs with antiangiogenic activity are currently approved as anticancer therapies axitinib (Inlyta), cabozantinib (Cometriq), pazopanib (Votrient), regorafenib (Stivarga), sorafenib (Nexavar), sunitinib (Sutent), and vandetanib (Caprelsa).

Axitinib (Inlyta)

Oral multikinase inhibitor that targets VEGFR-1, VEGFR -2, VEGFR -3

Indication: Advanced renal cell carcinoma after failure of one prior systemic therapy.

Cabozantinib (Cometriq)

Small molecule tyrosine kinase inhibitor of c-Met and VEGFR2.

Indication: Progressive, metastatic medullary thyroid cancer.

Pazopanib (Votrient)

Small molecule TK inhibitor of VEGF, PDGFR and c-kit

Indication: Advanced renal cell carcinoma.

Regorafenib (Stivarga)

Oral multikinase inhibitor that targets VEGFR-1, -2, -3, TIE2, PDGFR, and FGFR, KIT, RET, RAF, BRAF, and BRAFV600E.

Indication: Advanced metastatic colorectal cancer (mCRC) that has progressed or has recurred after multiple treatments.

Sorafenib (Nexavar)

Small molecule TK inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-ß, and Raf-1.

Indications

- Treatment of advanced renal cell carcinoma.
- Treatment of unresectable hepatocellular carcinoma.
- Treatment of locally recurrent or metastatic, progressive, differentiated thyroid carcinoma (DTC) refractory to radioactive iodine.

Sunitinib (Sutent)

Small molecule TK inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR- ß, and RET.

Indications

- Advanced renal cell carcinoma, GIST, pancreatic neuroendocrine tumors
- Treatment of gastrointestinal stromal tumor (GIST) after disease progression on or intolerance to imatinib mesylate.
- Progressive neuroendocrine cancerous tumors located in the pancreas that cannot be removed by surgery or that have spread to other parts of the body (metastatic).

Vandetanib (Caprelsa)

Small molecule TK inhibitor of VEGFR and EGFR

Indications

- Late-stage (metastatic) medullary thyroid cancer in adult patients who are ineligible for surgery.

Inhibitors of mTOR

Two mTOR inhibitors, temsirolimus (Torisel) and everolimus (Afinitor), are currently approved as anti-cancer therapy.

Temsirolimus (Torisel)

A small molecule inhibitor of mTOR (mammalian target of rapamycin), part of the PI3 kinase/AKT pathway involved in tumor cell proliferation and angiogenesis.

Indications: Advanced renal cell carcinoma, relapsed or refractory mantle cell lymphoma/Non-Hodgkins Lymphoma.

Everolimus (Afinitor)

A small molecule inhibitor of mTOR (mammalian target of rapamycin), part of the PI3 kinase/AKT pathway involved in tumor cell proliferation and angiogenesis.

Indications

- Advanced renal cell carcinoma, pancreatic neuroendocrine tumors, subependymal giant cell astrocytoma (SEGA).
- After failure of treatment with sunitinib or sorafenib.
- Treatment of progressive neuroendocrine tumors of pancreatic origin (PNET) in patients with unresectable, locally advanced, or metastatic disease.
- Patients with subependymal giant cell astrocytoma (SEGA) who require therapeutic intervention but are not candidates for curative surgical resection.

Other Antiangiogenic Agents

Interferon alfa (Intron® A and Roferon®)

An endogenous cytokine with antiangiogenic activity.

Indications: Hairy Cell Leukemia, Malignant Melanoma, Follicular Lymphoma, AIDS-Related Kaposi's Sarcoma.

Lenalidomide (Revlimid®)

Possesses immunomodulatory, anti-inflammatory, and antiangiogenic properties, although the precise mechanisms of action are not fully understood.

Indications

- Myelodisplastic Syndrome associated with 5q deletion, Multiple myeloma.
- Treatment of multiple myeloma in combination with dexamethasone in patients who have received at least one prior therapy.

Thalidomide (Thalomid®)

Possesses immunomodulatory, anti-inflammatory, and antiangiogenic properties, although the precise mechanisms of action are not fully understood.

Indications

- Multiple myeloma.
- Administered in combination with dexamethasone in patients with newly diagnosed multiple myeloma.

rhEndostatin

Endogenous angiogenesis inhibitor; recombinant protein; blocks VEGF-induced tyrosine phosphorylation of KDR-Flk-1 in endothelial cells, and down regulates MMP-2/9.

Indications: Non-small cell lung cancer (NSCLC) (http://www.angio.org/learn/treatments/).

Limitations of anti-angiogenesis therapy

In spite of this undeniable success, current anti-angiogenic therapies have revealed some limitations and several unanticipated problems are emerging.

1. In most tumours (with the exception of renal cell carcinoma), anti-angiogenesis treatment requires association with chemotherapy.

2. In most patients, addition of anti-angiogenic therapy to current standard therapies provides only limited overall survival benefits.
3. An unexpected problem of antiangiogenesis therapy in cancer is the development of resistance. This seems to be common to all currently clinically used anti-angiogenic drugs.
4. There is mounting evidence suggesting that anti-VEGF therapy (or anti-angiogenic therapy at large) may paradoxically enhance tumour progression by promoting an invasive phenotype that allows the tumour to escape angiogenesis inhibition.
5. An anti-angiogenic drug would probably stop the normal reproductive cycle in women and would be very dangerous to give to pregnant women.
6. High dosages are necessary to suppress tumor growth.
7. Other disadvantages of antiangiogenic protein therapy include the need for repeated injections and prolonged treatment.

In a recent investigation, unexpected side effects with combination of bevacizumab (anti- VEGF agent) and radiotherapy. Normal tissues toxicity was found to be triggered by the combined anti-angiogenic and radiation therapy.

Diabetic Retinopathy

Diabetic Retinopathy **(**DR) is one of the most serious complications of diabetes. Both type-1 (IDDM) and type-2 diabetes (NIDDM) may lead to blindness due to retinopathy, with type-1 being more Diabetic retinopathy (DR) is a complication of diabetes mellitus (DM) that affects the blood vessels of the retina and leads to blindness. The progression of retinopathy is gradual, advancing from mild abnormalities (characterized by increased vascular permeability) to moderate and severe non-proliferative DR (characterized by the growth of new blood vessels on the retina and posterior surface of the vitreous (Nakazawa 2009).

Pathology and etiology of diabetic retinopathy

Abnormality in functions due to alterations in chemical, molecular biology or retinal physiology in the blood retinal barrier leads to early DR. The chemical changes eventually induce permeability, surface- adhesive, structural and metabolic changes to cells of the retinal capillary wall. This malfunction is the result of the toxicity of high glucose concentrations (hyperglycaemia) as well as the toxic effects of by-products from stressed metabolic pathways. The affected proteins have structural, catalytic or mediator functions; thus there are diverse physical manifestations of early diabetic eye disease. These small symptoms in turn give rise to more serious manifestations of DR as the retina attempts to compensate. Auto-immune or viral destruction of the pancreatic β-cells, leading to insulin deficiency, causes Type I diabetes (Forrester and Knott, 1997). Insulin resistance of the cells leads to Type II diabetes. The retinal consequences of both these types (type-1 and 2 diabetes) are similar. Any variability between the consequences arises out of the different time of diabetes diagnosis. Type II diabetics often have sub- clinical disease for many years before receiving health care, whereas the symptoms of type I diabetics are acutely manifested in other organs. The myriad of consequences of hyperglycemia is the increased viscosity of diabetic blood, which is thought to exacerbate the hyperglycemic damage to the endothelial cells and basement membrane. The appearance of fenestrae and the lack of tight junctions influence the permeability of the vessel walls (Ishibashi and Inomata, 1993). This toxic or bio- chemical insult can lead to complete endothelial and pericyte cell loss, so that the capillaries become acellular or composed of the basement membrane alone (Stitt et al., 1995). The pericyte contractile cells, being only a partial structural component of the capillaries, are believed be regulatory in nature. Loss of pericytes cells combined with the loss of smooth muscle mass in the larger retinal vessels may increase the haemodynamic pressure, which causes distension and ballooning of capillaries also known as Microaneurysms or MA. The damaged

endothelium attracts and adheres to the mobile and inflammatory cells of the blood, which results in capillary blockage. MAs are the indication of abortive new vessel growth attempting to re-canalize or re-perfuse the blocked vessel (Forrester and Knott, 1997). MAs represent one of the first clear and easily perceptible changes in microvascular morphology. The consequences of pericyte loss, endothelial cell damage and MA formation (potentially coupled with impairment of RPE function) are the startingpoint for the development of DR.

Terms Related To Diabetic Retinopathy:

i. **Microaneurysms**: These are the first clinical abnormality to be noticed in the eye. They may appear in isolation or in clusters as tiny, dark red spots or looking like tiny haemorrhages within the light sensitive retina. Their sizes ranges from 10-100 microns i.e. less than 1/12th the diameter of an average optics disc and are circular in shape (The Berries: *Diabetic Retinopathy*,: http://www.theberries.ns.ca/ARchives/2006Winter/diabetic_retinopathy.html), at this stage, the disease is not eye threatening.

ii. **Haemorrhages**: Occurs in the deeper layers of the retina and are often called 'blot' haemorrhages because of their round shape.

iii. **Hard exudates**: These are one of the main characteristics of diabetic retinopathy and can vary in size from tiny specks to large patches with clear edges. As well as blood, fluid that is rich in fat and protein is contained in the eye and this is what leaks out to form the exudates. These can impair vision by preventing light from reaching the retina.

iv. **Soft exudates**: These are often called 'cotton wool spots' and are more often seen in advanced retinopathy.

v. Neovascularisation: This can be described as abnormal growth of blood vessels in areas of the eye including the retina and is associated with vision loss. This occurs in response to ischemia, or diminished blood flow to ocular tissues. If these abnormal blood vessels grow around the pupil, glaucoma can result from the increasing pressure within the eye. These new

blood vessels have weaker walls and may break and bleed, or cause scar tissue to grow that can pull the retina away from the back of the eye. When the retina is pulled away it is called a retinal detachment and if left untreated, a retinal detachment can cause severe vision loss, including blindness. Leaking blood can cloud the vitreous (the clear, jelly-like substance that fills the eye) and block the light passing through the pupil to the retina, causing blurred and distorted images. In more advanced proliferate retinopathy; diabetic fibrous or scar tissue can form on the retina (Vallabha et al., 2004).

Diabetic retinopathy has four stages or severity levels which are summarized in Table1

1. **Mild Nonproliferative Diabetic Retinopathy (NPDR).** At this earliest stage, small areas of balloon-like bulges called microaneurysms may protrude from the walls of the retina's tiny blood vessels.

2. **Moderate NPDR.** As the disease progresses, some blood vessels that nourish the retina are blocked

3. **Severe NPDR.** Many more blood vessels are blocked, depriving several areas of the retina with their blood supply.

4. **Proliferative Diabetic Retinopathy (PDR).** At this advanced stage, the retina sends signals for nourishment, triggering the growth of new blood vessels that are abnormal and fragile. The new blood vessels grow along the retina and toward the vitreous, the gel-like fluid that fills the inside of the eye. They may leak blood into the vitreous, causing severe vision loss and blindness.

The classic retinal microvascular signs of nonproliferative diabetic retinopathy are microaneurysms, hemorrhages, hard exudates (lipid deposits), cotton wool spots (accumulations of axoplasmic debris within adjacent bundles of ganglion cell axons), venous dilation and beading, and intraretinal microvascular abnormalities

Table 2. International Clinical Diabetic Retinopathy Disease Severity Scale

Proposed disease severity level	Findings observable upon dilated ophthalmoscopy
Mild NPDR	Microaneurysms only
Moderate NPDR	More than just microaneurysms but less than severe NPDR
Severe NPDR	No signs of PDR, with any of the following: 1. More than 20 intraretinal hemorrhages in each of four quadrants 2. Definite venous beading in two or more quadrants 3. Prominent intraretinal microvascular abnormalities in one or more quadrants
PDR	Neovascularization and/or vitreous/preretinal hemorrhage

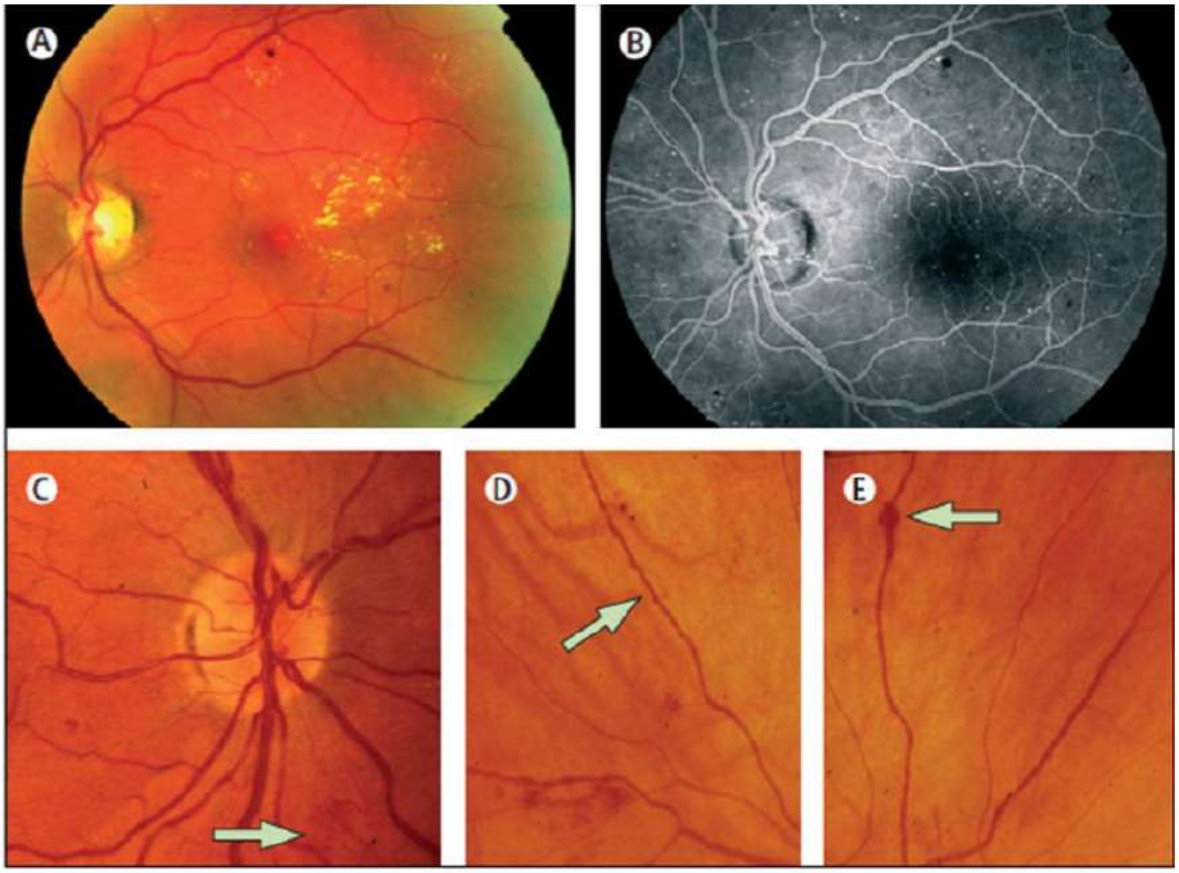


Figure. 14 Non-proliferative diabetic retinopathy.

Non-proliferative diabetic retinopathy. Cardinal signs are retinal microaneurysms, hemorrhages, and hard exudates (A and B); and intraretinal microvascular

abnormalities (C, arrow); venous beading (D, arrow); and venous loop formation (E, arrow).

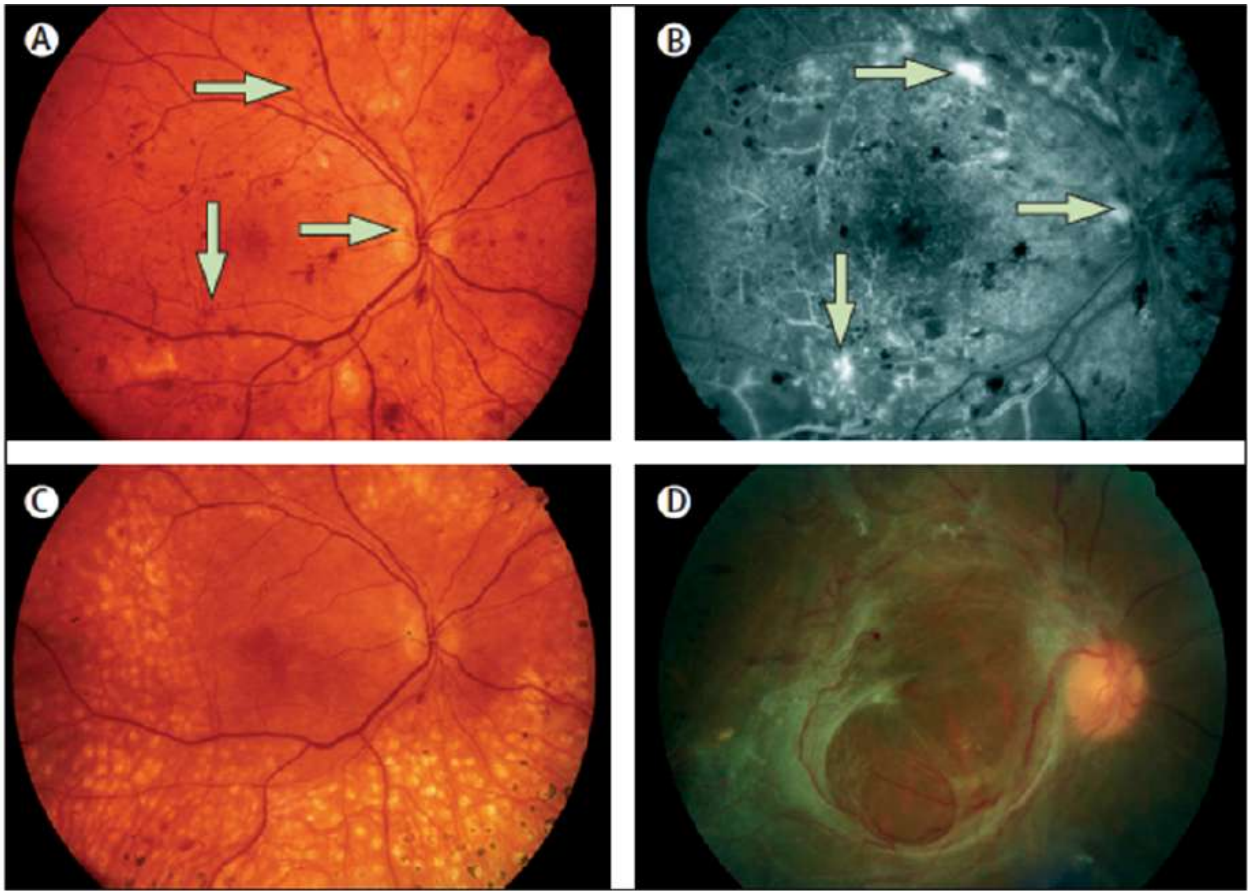


Fig: 15 Proliferative diabetic retinopathy.

Proliferative diabetic retinopathy. Neovascularization, a hallmark of proliferative diabetic retinopathy (A, arrows), which can be identified on fluorescein retinal angiogram (B, arrows); resolution of retinopathy with panretinal photocoagulation (C); progression of retinopathy without treatment to fibroproliferative disease (D) (Cheung et al. 2010).

Causes of Vision Loss and Risk Factors in diabetic retinopathy

Blood vessels damaged from diabetic retinopathy can cause vision loss in two ways

1. Fluid can leak into the macula, the area of the retina that is responsible for sharp, clear, central vision and allows us to see colors and fine detail. The fluid makes the macula swell, blurring vision. This condition is called macular edema (Ferris and Patz 1984). It can occur at any stage of diabetic retinopathy, although it is more likely to occur as the disease progresses. About half of the people with proliferative diabetic retinopathy also have macular edema.

2. Fragile, abnormal blood vessels can develop and leak blood into the center of the eye, blocking vision. This is proliferative diabetic retinopathy and is the fourth and most advanced stage of the disease. Other complications include detachment of the retina due to scar tissue formation and the development of glaucoma, an eye disease causing progressive damage to the optic nerve. In cases of proliferative diabetic retinopathy, the cause of this nerve damage is extremely high pressure in the eye. If left untreated, proliferative diabetic retinopathy can cause severe vision loss and even blindness.

All people with diabetes–both type 1 and type 2–are at risk for the development of diabetic retinopathy. The longer the patients have diabetes, the more likely they will develop diabetic retinopathy. Between 40 to 45 percent of Americans diagnosed with diabetes have some stage of diabetic retinopathy (http://www.nei.nih.gov/health/diabetic/retinopathy; Kempen et al. 2004). People with other medical conditions such as high blood pressure and high cholesterol are at greater risk (http://www.aoa.org/patients-and-public/eye-and-visionproblems/ glossary-of-eye-and-vision-conditions/diabetic-retinopathy?sso=y). Pregnant women face a higher risk for developing diabetes and diabetic retinopathy **(**Best and Chakravarthy 1997). If gestational diabetes develops, the patients are at much higher risk of developing diabetes as they age. Everyone with diabetes is recommended to get a comprehensive dilated eye examination at least once a year, since macula edema and proliferative diabetic retinopathy can develop without symptoms, such that patients are at high risk for vision loss.

Current Treatments of Diabetic Retinopathy

Treatment of diabetic retinopathy depends on the stage of the disease. During the first three stages of diabetic retinopathy, no treatment is needed, unless macular edema occurs.

Laser Surgery

If the disease advances, leakage of fluid from blood vessels can lead to macular edema, which is treated with laser surgery. This procedure is called focal laser treatment (photocoagulation) (http://www.nei.nih.gov/health/diabetic/retinopathy; http://www.aoa.org/patients-and-public/eye-and-visionproblems/glossary-of-eye-and-vision-conditions/diabetic retinopathy?sso=y). Up to several hundred small laser burns are created in areas of the retina with abnormal blood vessels to try to seal the leaks and reduce the amount of fluid in the retina. A patient may need focal laser surgery more than once to control the leaking fluid. When blood vessel growth is more widespread throughout the retina, as in proliferative diabetic retinopathy, a laser surgery called scatter laser treatment is needed (http://www.nei.nih.gov/health/diabetic/retinopathy; Neubauer and Ulbig 2007) 1,000 to 2,000 scattered laser burns are created in the areas of the retina away from the macula, causing the abnormal blood vessels to shrink and disappear. Because a high number of laser burns are necessary, two or more sessions usually are required to complete treatment. With this procedure, peripheral vision may be partially lost in order to preserve central vision. Scatter laser treatment may slightly reduce color vision and night vision.

Vitrectomy

In more advanced cases such as severe bleeding, a surgical procedure called a vitrectomy may be needed to restore sight by removing significant amount of blood from the center of the eye (vitreous gel) (http://www.nei.nih.gov/health/diabetic/retinopathy; Smiddy and Flynn 1999). Retinal detachment, defined as separation of the light-receiving lining in the back of the eye, resulting from diabetic retinopathy, may also require surgical repair **(**http://www.aoa.org/patients-and-public/eye-and-visionproblems/glossary-of-eye-and-vision-

conditions/diabetic-retinopathy?sso=y). A vitrectomy is performed under either local or general anesthesia. A doctor makes a tiny incision in the eye of a patient. Next, a small instrument is used to remove the vitreous gel that is clouded with blood and replace it with a salt solution to maintain the normal shape and health of the eye. Since the vitreous gel is mostly water, the patient will notice no change between the salt solution and the original vitreous gel. The patient's eye will be red and sensitive. The patient will need to wear an eye patch for a few days or weeks to protect the eye, and also need to use medicated eye drops to protect against infection.

Anti-VEGF Therapy

Anti-VEGF therapies are important in the treatment of diabetic retinopathy. They can involve monoclonal antibodies such as bevacizumab, antibody derivatives such as ranibizumab, or orally-available small molecules that inhibit the tyrosine kinases stimulated by VEGF. Both antibody-based compounds are commercialized. The efficacy of treatment with the anti-VEGF agents ranibizumab and bevacizumab indicates that VEGF contributes to the pathogenesis of diabetic macular edema and reflects successful translational research.

Natural resources for the exploration of anti-angiogenic substances

For over 40 years, natural products have served us well in combating cancer. The main sources of these successful compounds are microbes and plants from the terrestrial and marine environments.

A variety of antiangiogenic substances have also been isolated from natural sources. Fumagillin, produced by *Aspergillus fumigatis*, was one of the first agents found to act as an anti-angiogenesis compound. Other antiangiogenic substances from natural sources are shark cartilage, curcumin, the omega-3 and omega-6 fatty acids, green tea, licorice, quercetin, squalamine, and vitamin D3 (Marwick, 2001). Apart from shark cartilage, some marine

natural compounds from sponges and sponge-associated bacteria have also been reported to posses antiangiogenic potential (Marwick, 2001).

Emerging role of medicinal plants in angiogenesis inhibition

Plants have a long history of use in the treatment of cancer. Three quarters of anti-tumour compounds used in medicine are natural products or related to them. Of the 140 anti-cancer agents approved since 1940 and available for use, over 60% can be traced to a natural product. Of the 126 small molecules among them, 67% are natural in origin (Cragg and Newman, 2005). In 2000, 57% of all drugs in clinical trials for cancer were either natural products or their derivatives. From 1981 to 2002, natural products were the basis of 74% of all new chemical entities for cancer. Of 155 FDA-approved small molecule anti-cancer drugs, 47% were natural products.

Since angiogenesis plays a prominent role in tumour growth and metastasis, inhibition of angiogenesis is considered as an important strategy for cancer therapy. Studies on a global scale now focus to identify and develop anti-angiogenic factors to prevent growth and metastasis of tumour. Thus identification of a anti-angiogenic agent from natural sources that effectively act at various steps of angiogenic cascade could be of great clinical significance. Angiogenesis inhibition from plant sources are matter of great interest in recent times. Accordingly, numerous bioactive plant-derived compounds have been tested for their antiangiogenic potential. Systematic review shows that, many Indian Medicinal plants possess anti –VEGF activity.

With more than $4 billion invested in the research and development of antiangiogenic medicines, something has to come of this massively-financed area of research. The natural sources might have an important role in development of more potent, less toxic

and cheapest antiangiogenic agents. Anti-angiogenic agent from natural sources may complement the efficacy of chemotherapy and radiotherapy without much toxicity.

Table.3 List of Indian medicinal plants with anti-VEGF activity (Kumar et al. 2013)

Latin name	Family
Tinospora cordifolia	Menispermaceae
Tinospora cordifolia	Menispermaceae
Ocimum sanctum	Lamiacea
Azadirachta indica	Meliaceae
Azadirachta indica	Meliaceae
Calotropis procera	Apocynaceae
Withania somnifera	Solanaceae
Withania somnifera	Solanaceae
Curcuma longa	Zingiberaceae
Curcuma longa	Zingiberaceae
Commiphora mukul	Burseraceae
Piper longum	Piperaceae
Andrographis paniculata	Acanthaceae
Peganum harmala	Nitrariaceae
Vernonia cinerea	Asteraceae
Boswellia serrata	Burseraceae

METHODS FOR ASSAYING ANGIOGENESIS *IN-VITRO* AND *IN-VIVO*

One of the most important technical challenges in study of angiogenesis is selection of the appropriate assay. There are increasing number of angiogenesis assays being described both *in-vitro* and *in- vivo* (Staton *et al*., 2004**)**.

***In-vitro* assay of angiogenesis**

1. Endothelial cells *in-vitro*
2. Assays for endothelial cell migration
3. Differentiation assays
4. Organ culture assay

***In-vivo* assay of angiogenesis**

1. Implantation of sponges and polymers
2. Chick chorioallantoic membrane assay
3. Corneal angiogenesis assay
4. Dorsal air sac model
5. Chamber assays
6. Tumour models
7. Angiomouse
8. Zebrafish assay

Chick chorioallantoic membrane assay

The Chick chorioallantoic membrane (CAM) assay is probably the most widely used *in-vivo* assay for studying angiogenesis (Nguyen *et al*., 1994).The test substance is prepared either in slow-release polymer pellets, absorbed by gelatin sponges, or air-dried onto plastic discs; these are then implanted onto the CAM through a window cut carefully in the eggshell. The lack a mature, immune system in 7-8 day old chick embryos allows for the study of

tumour induced angiogenesis (Folkman, 1975). The angiogenic effects can be measured by counting the number of blood vessels in a given area using a stereomicroscope.

The CAM assay is relatively simple and inexpensive *in-vivo* assay suitable for large scale screening.

Chorioallantoic membrane

In the chick embryo, the chorioallantois is formed between days 4 and 5 of development, when the outer mesodermal layer of the allantois fuses with the mesodermal lining of the chorion, and a network of blood vessels is gradually formed between the two layers. The central portion of the CAM is fully developed by day 8 to 10 at which time it becomes capable of sustaining tissue grafts, while the outskirts of the CAM are still developing and expanding until the CAM fully envelopes the embryo at day 12 of incubation. Histologically, the CAM consists of three germ layers, that is, ectoderm, mesoderm, and endoderm. The ectoderm faces the shell membrane and is underlined by the respiratory capillary plexus, which starts to form between days 5 and 6 of embryonic development by both angiogenesis and vasculogenesis (Melkonian *et al.*, 2002). This capillary plexus is very dense and appears as a honeycomb network of tiny capillaries originating from terminal capillaries. The mesoderm of the chorioallantois is a collagen-rich embryonic connective tissue transversed by blood vessels belonging to the arteriolar and venous systems. The mesoderm is underlined with a thin endoderm layer, which separates the CAM from the allantoic cavity.

Until day 11 or 12 of chick embryo development, the blood vessel system of the CAM is highly angiogenic, that is, undergoing maturation through a constant generation of new blood vessels as well as establishment of new blood vessel anastomoses. Therefore, between day 8 and day 10, the developing CAM vasculature is ready to sprout in response to additional proangiogenic stimuli and, in turn, is very responsive to antiangiogenic factors. This feature renders the chick embryo CAM models well suited for experimental validation

of pro and anti-angiogenic compounds. In addition, the chick embryo is naturally immunoincompetent until embryonic day 17, thus allowing for grafting of cells of different species origin, such as human tumor cells, and therefore providing a useful tool for analysis of the proangiogenic potential of test cells.

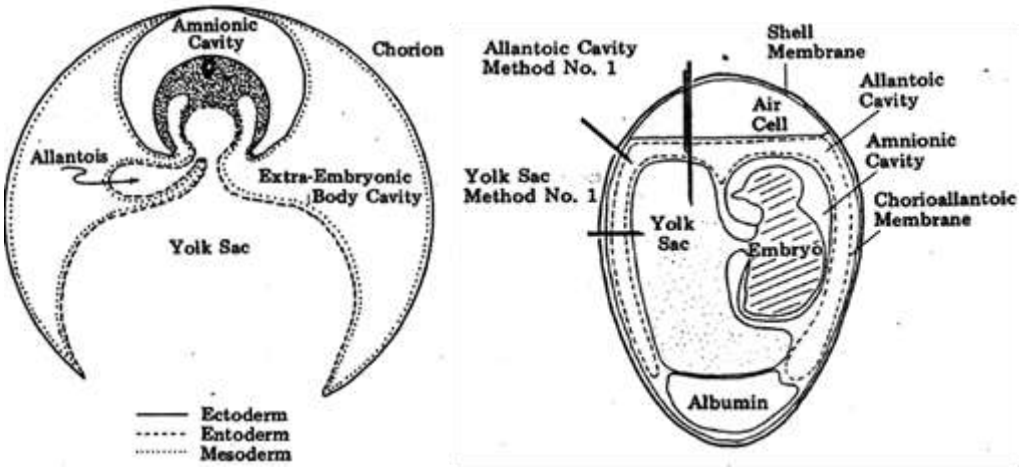


Figure.16 Development of Chorioallantoic membrane

REVIEW OF LITERATURE

PLANT PROFILE

Punica granatum tree

Root part of *Punica granatum*

Dried roots of *Punica granatum*

Fig:17 Tree, root part and dried roots of *punica granatum*

Taxonomical classification

Kingdom - Plantae plant

Subkingdom - Trachebionta –vascular plants

Superdivision - Spermatophyta-seed plants

Division - Magnoliphyta –flowering plants

Class - Magnolipsida- dicotyledons

Subclass - Rosidae

Order - Ryrtales

Family - Punicaceae pomegranate family

Genus - Punica L. Pomegranate

Species - *Punica granatum* L. pomegranate

Table 4: VERNACULAR NAMES

arabic	Gulnar	Philipin	Grande
assames	Dalim	punjabi	Da,danu
Bengali	Dalimgachh	Russian	Granatik
english	Pomegranate	Sanskrit	Bijapura
Hindi	Anar	Spanish	Grando
Italian	Melogrante	Swedish	Granated
kannada	Daadima	Tamil	Madulai
malayalam	Urumampalam	Telugu	Karakamu
Marathi	Dalimba	Tibetian	Se bru
persian	Darakhte-gulnar	urdu	Anarmitha

Description

The species is globally distributed in South Europe, Pakistan, India, West Asia. Within India, it is wild in the Western Himalayas between on altitude range of 900-1800 m., and is cultivated throughout the country for its fruits.

A shrub or small tree growing 6 to 10 m (20 to 33 ft) high, the pomegranate has multiple spiny branches and is extremely long-lived, with some specimens in France surviving for 200 years.

Leaves

Punica granatum leaves are opposite or sub-opposite glossy, narrow oblong, entire, 3-7 cm (1.2-2.8 inch) long and 2 cm broad. The flowers are bright red and 3cm in diameter, with three to seven petals. Some varieties are grown for the flowers alone.

Blossoms

Produced in summer where rainfall is minimal during late summer.

Flower

Colour: bright red with 5 to 8 crumpled petals which ersists in the fruit.

Size : 3 cm in diameter.

Fruit

The edible fruit is a berry, intermediate in size between a lemon and a grapefruit, 5-12 cm (2.0-4.7 in) in diameter with a rounded shape and thick, reddish skin. The number of seeds in a pomegranate can vary from 200 to about 1400. Each seed has a surrounding water laden pulp- the edible sarcotesta that forms from the seed coat- ranging in color from white to deep red or purple. The seeds are exarillate i.e. unlike some other species in the order, myrtales, no aril is present. The sarcotesta of pomegranate seeds consists of epidermis cells derived from the integument. The seeds are embedded in a white, spongy, astringent membrane.

Pomegranate juice, obtained by compressing the seeds, causes a deep red stain which is difficult to remove. The pigmentation of pomegranate juice results from the presence of anthocyanins and ellagitannins (Dipak et al. 2012).

Traditional uses

Heart problem

Frequent intake of pomegranate juice can maintain good folw of the blood in the body. Along with this, it decreases the risk of heart attack and heart strokes.

Stomach disorder

Pomegranate peel, bark and leaves are used to calm the stomach disorder or diarrhea triggered due to any kind of digestive problems. Drinking tea made from the leaves of this fruit helps in curing digestive problems. Pomegranate juice is also used for handling problems of dysentery and cholera.

Dental care

The best benefit of pomegranate is that its juice, along with its antibacterial and anti viral properties, helps to reduce the effects of dental plaque.

Cancer

Pomegranate consists of advanced level of antioxidants called flavonoids. These flavonoids are thought to be effective in counteracting various cancer radicals. The individuals that face high risk of prostate and breast cancer should start drinking the juice of this fruit, as this will help them to reduce further risk of developing cancer. Regular consumption of pomegranates can reduce the PSA levels in the body and helps to fight the existing cancer cells in the body.

Osteoarthritis

Pomegranate minimizes the illness triggered in various forms like atherosclerosis and osteoarthritis. The loss that is triggered due to the thickening and solidifying of the arterial walls and in cartilage and joints can be cured by consuming this fruit. Also, pomegranate is capable of preventing the creation of minerals that are liable for breaking down the connective tissues.

Diabetic anemia

Consuming of pomegranate fruit juice by a diabetic patient can prevent coronary illness. Along with this, there is a slowdown in solidifying of the bloodstream, which can fuel non-occurrence of various heart disease.

Anemia

Healthy blood flow can be maintained in the body by consuming this fruit in any form. Pomegranate seed extract supplies iron to blood and thus help to decrease the anemic symptoms including fatigue,wooziness and weakness and hear loss. (Bhowmik et al. 2013)

OTHER ADVANTAGEOUS FORMS OF

Pomegranate

With the passage of time, more and more people have started acknowledging the importanceofconsumingpomegranates.Thereareotheradvantagetoo,like pomegranate reduces the likelihood of having premature infants and it is also beneficial for the expected mothers to avoid having low weight infants during birth.

Pomegranate seed extract also reduces the likelihood of creating Alzheimer's disease among the elderly. It helps controlling aging issues like wrinkles and thus, facilitates youthful and glowing skin. Other than this, it allows a woman to overcome from her depression interval , especially from the menopause period.

The pomegranate fruit juice is also known to be very helpful in treating issues of erectile dysfunctions. It is a good natural aphrodisiac and improves sperm count and semen quality. The astringent features of the flower juice, rind and tree bark are considered valuable for a wide range of purposes, such as stopping nose bleeds and gum bleeds, toning skin (after mixing with mustard oil) firming-up sagging breasts and treating hemorrhoid. Pomegranate

seed (of specific fruits trains) is also used as eye drops as it is believed to slow the development of cataracts. Pomegranate is used as a gargle for a sore throat,and it is applied to the epidermis to cure hemorrhoid flare-ups. It cleanses and clarifies oral cavity, throat, esophagus stomach and chest.

Health benefits of pomegranate

Free radicals

Pomegranates are a rich source of anti oxidants that helps to protect our body's cells from free radicals, which cause premature aging. Free radicals are formed due to exposure to the sun and harmful toxins from the environment.

Pomegranate is natural blood thinners:

Prevents blood clots in the heart and arteries, also urinary retention. The seeds prevent your blood platelets from coagulating and forming clots.

Arthritis prevention:

Pomegranate can reduce the damage on the cartilage for those hit with arthritis. This fruit has the ability to lessen the inflammation and fights the enzymes that destroy the cartilage.

Help in erectile dysfunction:

Pomegranate juice can improve erectile dysfunction only moderately.

Prostrate cancer and heart diseases:

Two separate studies claim that pomegranate juice helps fight prostate cancer. In one lab experiment, the juices "slowed the growth of the cultured cancer cells and promoted cell

death". In the second experiment, pomegranate juice improved the condition of the blood, hence improving the health of individuals down with cardiovascular diseases.

Prevention of atherosclerosis:

Pomegranates prevent the hardening of the artery walls with excess fat, leaving your arteries fat free and pumping with antioxidants.

Medicinal Properties of Pomegranates

The word “Pomegranate" (*Punica granatum*) comes from the Latin for "fruit of many seeds."

In folk medicine, the fruit's astringent properties have been used to treat various ailments (cuts,sore throats, tapeworms, dysentery, and gum disease). Pomegranate juice is marketed in the United States as a major source of anti oxidant nutrients that protect against heart disease and other ailments.

Recent research has focused on its potential use as a treatment for cardio vascular disease, diabetes, and various forms of cancer. The author examines those properties of the pomegranate, as well as its history and nutritional and chemical makeup.

Pomegranates are believed to be native to the areas from eastern Iran through northern India, says the author. More than adozen cultivars of the fruit ("Wonderful" being the leading commercial cultivar in the United States) have been grown commercially in California's San Joaquin Valley since its introduction by Spanish settlers in the late 18thcentury.

Pomegranates are a good source of vitamin C, providing between 1020% of there commended daily allowance according to onesource1 and up to 40% according to another.

The potent antioxidant properties of the fruit have been attributed to its high content of soluble polyphenols.

When tested in vitro on normal and colon-cancer cell lines, the juice was found to have superior antioxidant, anti proliferative, and pro apoptotic effects compared with single purified active ingredients, probably the result of synergistic actions among the fruit's multiple compounds. Studies have shown that the antioxidant activity of the pomegranate flowers yielded activity two to three times the antioxidant potency of tea or red wine.

The author notes research suggesting that pomegranate juice maybe cardio protective, reducing risk factors (such as cholesterol accumulation, foam-cell formation in macrophages, and oxidized low-density lipoprotein [LDL]) without affecting native LDL. Cited by the author is an Israeli study in which 10patients with carotid artery stenosis (advanced plaque build-up in the arteries) drank pomegranate juice and experienced reduced blood pressure, LDL oxidation, and progression of carotid lesions at 1-year and 3-year study intervals. In a randomized, double-blinded, placebo-controlled study at the Preventive Medicine Research Institute in Sausalito, CA, pomegranate juice drinkers with coronary artery disease had a 17% improvement in blood flow compared with an 18% worsening in the control group.

The study team concluded that the antioxidants in the juice may help prevent the formation of fatty deposits on artery walls. In studies of the fruit's anticancer effects, pomegranate fruit extract (PFE) has been found to be chemo preventive in mouse mammary organ culture and in human breast cancer cells in vitro. In another study cited by the author, researchers at the University of Wisconsin in Madison found that PFE significantly reduced serum prostate specific antigen levels and inhibited proliferation of aggressive human prostate cancer cells in athymic mice.

Pomegranate extracts have exerted anti proliferative, anti estrogenic, and pro apoptotic actions on leukemia cells as well as breast- and prostate-cancer cells. Results of studies with diabetic patients have shown that supplementing the diet with pomegranate juice had beneficial antioxidant effects on macrophages, implying that it could reduce the development of atherosclerosis.

Australian researchers found that pomegranate flower extract reduced factors (hyperglycemia, hyperlipidemia, and a fatty heart) that can result in increased cardiac impairing fibrosis in patients with type 2 diabetes. Other studies have shown the benefits of pomegranate in promoting neurologic health ,maintaining joint integrity and function, exhibiting estrogenic properties, blocking herpes simplex virus replication and adsorption, enhancing immune function, treating periodontal disease, enhancing the activity of antibiotics used to treat methicillin -resistant and methicillin sensitive Staphylococcus aureus infections, and preventing smooth muscle dysfunction and fibrosis in erectile dysfunction. The author also mentions other uses of the fruit.

In Ayurvedic medicine, the astringent properties of pomegranates are linked with bone and cartilage build-up; in the cosmetic arena, fruit-peel extract has been shown to stimulate a type of pro collagen synthesis and inhibit a dermal degeneration process. The antioxidant, immune-boosting, and ante carcinogenic properties of the pomegranate, says the author, offers multiple potential medical applications (Bhowmik et al. 2013).

Medicinal Benefits

Pomegranate is a poly-vitamin, a unique fruit plant producing a wide spectrum of biologically active substances especially important in our present-day polluted environment. It helps in preventing the harmful effects of radioactive substances by producing biologically

active substances. Russians, after the deadly Chernobyl tragedy, used pomegranates to reduce the effect of radioactive substances.

In order to maintain the health and energy levels of astronomers, submariners and coal miners, they often consume pomegranate juice regularly. Pomegranate is loaded with tannins, anthocyanins, polyphenolic sand antioxidant vitamins, A, E and C, all of which have a health effect on the body. These elements work together to benefit the arteries, plus it keeps the cardiovascular system healthy which is the chief health benefit of Pomegranate.

It has also been found to increase levels of nitric oxide, which improve blood flow to the heart, reduce arterial plaque, reduce systolic blood pressure and help in curing erectile dysfunction. Other benefits include preventing premature aging, stroke, arthritis, Alzheimer's and even cancer. The juice of the red pomegranate has received attention for its rich flavor and health boosting properties. If you cut a pomegranate open, you will see the many tiny pomegranate" arils" or seeds that are contained inside.

The juice comes from the crushed seeds. Pomegranate juice has been shown to contain more antioxidants than most fruit juices, red wine or green tea, according to Health Castle.

Antimicrobial Properties

Drinking pomegranate juice has been shown to have antimicrobial properties against harm full bacteria that can exist in the stomach, such aseschericia Coli (e.Coli) or Bacillus subtilis, both of which can cause painful infections and serious stomach conditions.

Fighting Cancer

Pomegranate contains a number of beneficial antioxidants, including polyphenols, tannins and anthocyanins. Antioxidants protect against free radicals, which are by-products of cell oxidization. Free radicals are associated with causing a number of health problems, including breast, prostate and lung cancers.

Drinking pomegranate juice has been shown to shrink prostate tumors in mice, and this could be due to the fact that antioxidants contained within pomegranate juice help to fight against free radical damage. This also boosts immunity, which helps to lower the risk of cancer incidence.

Essential Vitamins and Minerals

Pomegranate juice is high in a number of vitamins and minerals, including 40 percent of the recommended daily allowance of Vitamin C perserving. Other essential vitamins and minerals include Vitamins A and E and folic acid. Meeting daily health requirements helps to enhance health and boost immunity.

Protects against Arthritis

Pomegranate juice contains an enzyme inhibitor that prevents enzymes from damaging cartilage in the body. This benefit helps to prevent the onset of or even symptoms associated with osteoarthritis.

Blood Thinner

Drinking pomegranate juice has been shown to act as a natural blood thinner. This helps to increase the flow of blood to the heart while also reducing arterial plaque. While

pomegranate juice alone would not act as a strong enough blood thinner on its own in those with heart disease, the juice can still have a beneficial effect on the heart. Pomegranate is a tree. Various parts of the tree and fruit are used to make medicine.

Pomegranate is used for many conditions, but so far, there isn't enough scientific evidence to rate pomegranate as effective for any of them. We do know, though, that pomegranate does not seem to be effective for reducing the symptoms of chronic obstructive (COPD) or improving breathing in people with this condition.

Pomegranate is used for conditions of the heart and blood vessels, including high blood pressure, congestive heart failure (CHF), heart attack, "hardening of the arteries" (atherosclerosis), and high cholesterol. It is also used for conditions of the digestive tract, including diarrhea, dysentery, and tapeworm and other intestinal parasites .Some people use pomegranate for flu, swelling of the lining of the mouth (stomatitis), gum disease, erectile dysfunction (ED), diabetes and a complication called acidosis, bleeding, and HIV disease. It is also used for preventing prostate cancer, obesity, and weight loss.

Some women use pomegranate to cause an abortion. Pomegranate is used as a gargle for sore throat , and it is applied to the skin to treat hemorrhoids. Pomegranate (*Punica granatum*) is unique among plants. The only other plant that is closely related is a small tree that grows only on an island in Yemen.

Pomegranate has been used for thousands of years to treat a wide variety of diseases. It is in Greek, Hebrew, Buddhist ,Islamic, and Christian mythology and writings. It is described in records dating from around 1500BC as a treatment for tapeworm and other parasites (Bhowmik et al. 2013)..

Medicinal Uses of *Punica granatum L*

(Pomegranate)

Pomegranate fruit juice is known as a delicacy and is made into excellent sherbet with the addition of water, sugar and taken internally, and some people use it in preparing ice-creams, jellies and marmalades.

Such juice of pomegranate fruits possesses diuretic, cooling effect, glucose, fructose, tannins, oxalic acid, and reduces thirst in cases of fevers, supplies the required minerals and helps the liver to preserve vitamin A. from the food, increases the body's resistance to T. B infection, and acts as a tonic for heart and kidney.

According to Indian Herbal System, all parts of pomegranate including roots, leaves, flowers, rind, seeds and the reddish brown bark are used medicinally. Pomegranate bark and root contains several alkaloids including iso pelletierine that fights against tapeworms.

Pomegranate bark, leaves, immature fruit and fruit rind extracts is given to combat diarrhea, dysentery and hemorrhages, whilst powdered flower buds acts as a remedy for nose bleeding. For bleeding piles; the bark decoction is very effective, and if combined with Holarrhena’s bark with a sip of honey it treats blood motions.

For threatened abortion; pomegranate leaves, sandal wood powder, curd and a sip of honey are useful .For gum bleeding and bleeding of the teeth; the fruit rind powder mixed with black pepper, common salt, and applied. Such preparation whitens teeth, strengthens gum and said to prevent pyorrhea. For urinary calculus; a teaspoonful of ground seeds along with a cup of gram soup taken internally For diarrhea, dysentery, nose bleeding, prolapsed rectum, leucorrhea, etc.; powdered dried rind with fenugreek decoction and a sip of honey are beneficial.

The flower bud can also be snuffed in case nose bleeding. For conjunctivitis; a paste of the leaves is applied on the red part of the eye. This is also beneficial in healing scabies, eczema, itchiness and ringworm. It has immuno- stimulatory, anti-oxidant ,anti-inflammatory anti-diabetic and anticancer. It is widely used in treating certain types of cancer including gleukemia, breast, prostate and colon cancer, dysentery, diarrhea, excessive bleeding, intestinal worms and parasites.

Recent Research

New therapies for preventing cancer may be on their way as scientists have identified components in pomegranate juice that inhibit the movement of cancer cells. Researchers at the University of California have found that these components also weaken cancer cells attraction to a chemical signal that promotes the metastasis of prostate cancer to the bone.

The research could lead to new therapies for preventing cancer metastasis .Manuela Martins-Green applied pomegranate juice on aboratory-cultured prostate cancer cells that were resistant to testosterone. The researchers found that the pomegranate juice treated tumour cells that had not died with the treatment showed increased cell adhesion and decreased cell migration (Bhowmik et al. 2013).

Chemical constituents

Alkaloids

It was indicated that alkaloids was present at the rate of 3% in the roots. It was also indicated that

- Pseudopelletierine
- Pelletierine

- Isopelletierine
- Methylpelletierine 1- pelletierine
- dl-pelletierine
- Methyl isopelletierines, were found in composition of the root, body and branch rinds of *Punica granatum*.

Tannins

It was stated that punicacortein A B C D in the structure of hydrolysable c-glycoside, which is a new ellagitannin, as well as punigluconin which contains one gluconic acid and also casuariline and casuarine were present in the fresh body roots of *Punica granatum*. Ellagitannins including punicalin and punicalagin.

Triterpenic acids

Presence of ursolic acid one of the compounds in triterpenic structure was determined in different sections of the pomegranate plant.

Biochemical constituents:

Three current research seems to indicate the most therapeutically beneficial pomegranate constituents are ellagic acid ellagitannins (including punicalagins), punicic acid, flavonoids, anthocyanindins, anthocyanins and estrogenic flavonols and flavones (Mohammed and Kashani 2012).

Scientific claims of *Punica granatum* L.

- **Jayakumar et al. 2012** evaluated the anticancer activity of *Punica granatum* rind extract against human lung cancer cell line A549. *Punica granatum* extract produced a dose dependent inhibitory effect on cell growth (IC50-80µg/ml) and significantly inhibited the growth of A549 cells.

- **Sajjad et al. 2015** evaluated the antitumor potential of *Punica granatum* peel extract. The antitumor activity was examined by using *Agrobacterium tumefaciens* At 10 strain and potato disc tumor assay technique and assessment were established on the basis of tumor inhibition of several concentration of extract (10ppm, 100pm,1000pm). Results discovered that different concentration of *Punica granatum* peel extract showed different % of inhibition. Increase in percentage inhibition of tumor were observed with increase of peel extract concentration. At 1000ppm, crude ethanolic extract (CEE) showed maximum inhibition 65%.

- **Dana et al. 2015** evaluated the anti-angiogenic and antiproliferative effects of black pomegranate peel extract (PPE) on melanoma cell line. Angiogenesis was investigated by matrigel assay. HUVECs, vascular endothelial growth factor (VEGF) mRNA expression was detected by quantitative reverse transcriptase–polymerase chain reaction (QRT-PCR) assay. VEGF concentration in culture medium of HUVECs was determined by enzyme-linked immunosorbent assay (ELISA). PPE showed positive anti proliferative effect on melanoma cells in a dose-dependent manner, but not on HUVECs. The matrigel assay results indicated that PPE significantly inhibited length, size and junction of the tube like structures. VEGF

mRNA expression and concentration levels in culture medium of PPE treated HUVECs reduced significantly in a concentration-dependent manner.

- **Qasim et al. 2013** evaluated the effect of pomegranate seed oil and tamoxifen on breast cancer tumor marker (CA 15 - 3), aromatase enzyme, triglyceride (TG), cholesterol, and lactate dehydrogenase (LDH) in mastectomy women. The results indicates tumor marker level was increase significantly in women without treament while tumor marker significally decreased in both pomegranate - tamoxifen combination. LDH level was decreased in both groups when compared with untreated group.

- **Vakili and Sumanth 2016** evaluated the anti-proliferative and apoptotic effects of *Punica granatum* L. and *Ziziphus mauritiana* against carcinoma cells *in-vitro* and *in-vivo*. *In-vitro* antioxidant activity of ethanol, aqueous and chloroform seed extracts of *Punica grantum* L and *Ziziphus mauritiana* showed that the ethanol extract of combination of both plants having significant Super oxide, Nitric oxide and ABTS scavenging activity with IC_{50} value of 32.79,31.95,78.85 µg/ml respectively. The MTT assay indicated that the percentage decrease of HeLa cells survival was found to be 18.68% in 100 µg of ethanol extract of combination of *Punica grantum* L. and *Ziziphus mauritiana* and it also stimulated caspase-3 protease by 17.9-fold increase in DEVD-pNA cleavage. The ethanol extracts of combination of both plants induced chromosomal aberrations such as spindle disturbance, bridge chromosome, sticky chromosomes, and polar deviations in root tips of onions and also showed root growth inhibition activity at part with colchicine as standard and these differences were statistically non-significant after 72h.Consequently. Synergic anticancer activity of ethanol extract of *Punica grantum* L and *Ziziphus mauritiana* was proved.

- **Dana et al. 2016** evaluated the role of peroxisome proliferator activated receptor alpha and gamma in anti-angiogenic effect of pomegranate peel extract (PPE). In this study the (PPARs) activation in the human umbilical resin endothelial cells (HUVECs) was evaluated. The mRNA expression level of vascular endothelial growth factor (VEGF) was detected by quantitative reverse transcription polymerase chain reaction (QRT-PCR). PPE significantly inhibited both tube formation (size, length and junction of tubes) and VEGF mRNA expression ($p<0.05$). Results showed that the anti-angiogenic effects of PPE were significantly reversed by PPAR antagonists. There was no difference between PPE Plus antagonists groups and control group. The results showed this effect of PPE could be mediated in part through PPAR dependent pathway.

- **Joseph et al. 2011** evaluated the antI-oxidant, anti-tumor and immunomodulatory properties of the polysaccharide (PSP001) isolated from fruit rind of *Punica granatum*. The anti- cancer properties of polysaccharide (PSP001) was evaluated on MCF-7 (breast cancer), KB (nasopharyngeal carcinoma) and K562(luekemia) cells by MTT assay. An IC50 value of 97.21 ± 1.06 µg/ml and 52.8 ± 0.9 µg/ml were obtained following 72 h incubation for MCF-7 and K562 cells, respectively. PSP001 showed *in-vitro* growth stimulatory effect on isolated normal lymphocytes, and a proliferative index of 1.21 ± 0.01 at a concentration of 1000 µg/ml, indicating immunomodulatory activity.

- **Seidi et al. 2016** evaluated anti-tumoral properties of the *Punica granatum* seed extract (PSE) in different human cancer cells. Anti-proliferative properties of seed extract of PSE from human cancer cells was studied by MTT assay with A549 (lung non small cell carcinoma), MCF-7 (breast adenocarcinoma) SKOV3 (ovarian cancer

cells), pc3 (prostate adenocarcinoma). Results were compared to negative control at all test dose. SKOV3 ovarian cells were the most responsive to the anti proliferative effects of PSE with maximum growth inhibition and A549 ells are least responsive for cytotoxic effects.

- **Khan et al. 2013** evaluated the effect of *Punica granatum* fruit extract on angiogenesis by using CAM assay and to verify either the extract is angiogenic or anti-angiogenic. A noticeable reduction in surface roughness of the blood vessels was observed. The diameter of tertiary, secondary and primary blood vessels was also reduced as compared to the blood vessels of control group CAM. The maximum effect was seen with 0.5% dilution. The study proved that *Punica granatum* (Pomegranate) fruit extract was anti-angiogenic and can be included in the studies for the development of new drug studies to treat cancer (as an anti-angiogenic agent).

- **Kawaii and Lansky 2004** evaluated the differentiation promoting activity of pomegranate fruit extract in HL-60 human promyelocytic leukemia cells. Flavonoid-rich fractions from fresh (J) and fermented (W) pomegranate juice and from an aqueous extraction of pomegranate pericarps (P) were tested for potential differentiation-promoting agents in human HL-60 promyelocytic leukemia cells. Four assays were used to assess differentiation: nitro blue tetrazolium reducing activity, nonspecific esterase activity, specific esterase activity, and phagocytic activity. In addition, the effect of these extracts on HL-60 proliferation was evaluated. Extracts W and P were strong promoters of differentiation in all settings, with extract J showing only a relatively mild differentiation-promoting effect. The extracts had proportional inhibitory effects on HL-60 cell proliferation.

- **Vakili and Sumanth 2016** evaluated *in-vivo* anti cancer activity of *Punica granatum* L and *Ziziphus mauritiana* using Ehrlich ascites carcinoma in Swiss albino mice. Experimental tumor was induced by inoculation of $1x10^6$ Ehrlich ascites carcinoma cells from the tumor bearing mice aseptically. A group of mice were administered aqueous, ethanol,chloroform extract of *Punica grantum* L of 200 mg/kg b.w, respectively, for nine days. Another group of mice were administered aqueous, ethanol, chloroform extract of *Ziziphus mauritiana* 200 mg/kg b.w, respectively for nine days. A group received combination combination of both plant extracts at a dose of 200 mg/kg. Change in body weight, survival time, ascites fluid volume and packed cell volume were noted. At the end of study, 6 animals from each group were sacrificed, blood samples were collected and WBC, RBC, Hb content was estimated. Ethanol extract of combination of *Ziziphus mauritiana* and *Punica grantum* showed significant reduction in body weight, tumor volume, packed cell volume and percentage increase in life span. Significant increase in RBC, Hb content and reduction in WBC count were observed.

- **Nair et al. 2011** evaluated the pomegranate extract (PE) on human pancreatic cancer cells. PAC-1 and As PC-1 human pancreatic cancer cell were used as *in vitro* models to test the effect of PE. PE treatment induced cell cycle arrest and inhibited cell proliferation in PANC-cells. PE was more effective in inhibiting the proliferation of PANC-1 cells than the clinically used dose of paclitaxel. Data suggest that PE is a promosing candidate for further preclinical testing for treatment of human pancreatic cancer.

- **Morrira et al. 2017** evaluated the anti-oxidant and cancer chemopreventive activities of Cistus and pomegranate polyphenols. Polyphenol rich extracts obtained from cistus

herb (*Cistus incanus* L.) and pomegranate peel (*Punica granatum* L.) exhibited significant antioxidant activity in V79 cell culture (Chinese hamster lung fibroblasts). Cistus extract reduced intracellular content of reactive oxygen species (ROS) by 30-40% and pomegranate extract by 29-36%. In human breast (MCF-7) and colon (LOVO) cancer cell lines cistus and pomegranate extracts decreased cancer cell growth both in drug-sensitive cells by 15-30% and in drug resistant (doxorubicin-resistant; DX) sublines by 5-20%. Significantly higher proapoptotic impact of the extracts was observed in drug-sensitive than in drug-resistant sublines. The results suggest potential usefulness of the tested polyphenol rich extracts in people exposed to oxidative stress.

- **Hora et al. 2003** evaluated the chemopreventive effects of pomegranate seed oil on skin tumor development in CD mice. Two groups consisting each of 30, 4-5-week-old, female CD (1) mice were used. Both groups had skin cancer initiated with an initial topical exposure of 7,12 dimethyl benzanthracene and with bi weekly promotion using 12-O-tetraxe decanoylphorbol 13-acetate (TPA). Tumor incidence, tumor multiplicity were measured. Tumor incidence, the number of mice containing at least one tumor, was 100% and 93%, and multiplicity, the average number of tumors per mouse, was 20.8 and 16.3 per mouse after 20 weeks of promotion in the control and pomegranate seed oil-treated groups, respectively.

  In a second experiment, two groups each consisting of three CD (1) mice were used to assess the effect of pomegranate seed oil on TPA-stimulated ornithine decarboxylase (ODC) activity, an important event in skin cancer promotion. Each group received a single topical application of TPA, with the experimental group receiving a topical treatment 1 h prior with 5% pomegranate seed oil. The mice were

killed 5 h later, and ODC activity was assessed by radiometric method. The experimental group showed a 17% reduction in ODC activity.

- **Modaeinama et al. 2015** evaluated the anti-tumoral properties of *Punica granatum* peel (PPE) extract on different human cancer cells. The anti proliferative properties of peel extraxt of human cancer cells were evaluated. The cytotoxicity of different dose of PPE was evaluated by MTT assay with A549 (lung non small cell cancer), MCF-7 (breast carcinoma) SKOV3 (ovarian cancer) and PC-3 (prostate adenocarcinoma) cells. In all studied cancer cells, PPE reduced the cell viablity to values below 40% even at the lowest doses. In all cases IC_{50} was determined at doses below 5 µg/ml. MCF-7 breast adenocarcinoma cell were the most responsive cells to anti proliferative effects of PPE with a maximum mean growth inhibition of 81% vs 69.9%, 69.4%, 79.3% and 77.5% in SKOV3, PC-3 and A549 cells, respectively. Low doses of PPE exerted potential anti proliferative effects in different human cancer cells and its seems that (MCF-7 breast adenocarcinoma) cells are the most cells and SKOV3 ovarian cancer cells the least responsive in this regard.

- **Adams et al. 2010** evaluated the pomegranate ellagitannin derived compounds on anti proliferative and anti aromatase activity in breast cancer cells *in vitro*. Estrogen stimulates the proliferation of breast cancer cell and growth of estrogen responsive tumors. A panel of ten ellagitannins (ET)-derived compounds including EA, gallagic acid (GA), and urolithins A and B (and their acetylated, methylated and sulfated analogs prepared in our laboratory) were examined for ability to inhibit aromatase activity and testosterone-induced breast cancer cell proliferation. Using a microsomal aromatase assay, the panel of ET-derived compounds were analyzed and it was identified that six compounds with anti-aromatase activity. Among these, urolithin B

(UB) was shown to most effectively inhibit aromatase activity in a live-cell assay. Proliferation assays also determined that UB significantly inhibited testosterone-induced MCF-7aro cell proliferation. These studies suggest that pomegranate ET derived compounds have potential for the prevention of estrogen responsive breast cancers.

- **Motaal, and Shanker 2011** evaluated anti-cancer and anti-oxidant activities of standarized whole fruit, pulp and peel extract of Egyptian pomegrante. An HPLC method was modified and validated for standardisation using ellagic acid (EA) as a marker. The peel showed the highest anti-oxidant activity of (IC50=0.50) ± 0.9mg/ml) compared to other two extract, as well as pronounced anti cancer activity of against MCF-7 human breast cancer cell and HCT-116 colon cancer cell with IC50 values 7.7 ± 0.01 and 9.3 ± 0.06µg/ml respectively. The standardized peel extract was formulated into capsules to develop natural pharmaceutical preparations.

AIM AND OBJECTIVE

Angiogenesis is a process of formation of new blood vessels from a pre-existing vasculature. It plays a vital role in embryonic development and numerous pathological conditions including cancer, rheumatoid arthritis, diabetes retinopathy, age related macular degeneration and neurological disorders such as Parkinsonism and Alzheimer's disease (Folkman 1990; Folkman 1995). In cancer, the growth and metastasis of tumour are dependent on angiogenesis (Hazel 2003). Cancer cells can generate various pro-angiogenic factors such VEGF, FGF, EGF. These factors promote the migration, proliferation and tube formation of endothelial cells which are essential steps for angiogenesis. The newly formed blood vessels can promote cancer growth by supplying nutrients, oxygen and most importantly facilitate cancer cell metastasis to other localities (Sassa and Hatta 2009; Homayouni 2009). In diabetic patients, hyperglycemia is the triggering factor for tissue alterations such as damage to capillary endothelial cells in the retina and BRB breakdown (Zhang et al. 2014**;** Brownlee 2005) and one of the key players is vascular endothelial growth factor (VEGF), which promotes angiogenesis, abnormal vascular permeability, and eventually, an inflammatory response. Thus interrupting the process of angiogenesis has become one of the promising approaches in the treatment and prevention of cancer progression and diabetic retinopathy.

Drug development from natural products has become a rapidly emerging and highly promising strategy to identify novel anti-angiogenic and anti-tumour agents. Plant derived compounds have played an important role in the development of several clinically useful anticancer agents. Over 62 % of biologically active substances currently used as anticancer agents are derived from natural sources, including plants, marine organism and microorganism. Today over 300 anti-angiogenic molecules targeting different signalling

pathways are being tested for their anticancer properties at preclinical and clinical stages (Sassa and Hatta, 2009; Homayouni, 2009). Although the results of clinical trials are encouraging the effects were modest (Lu and Bergers, 2013). Therefore, the search and discovery of novel anti-angiogenic principle that selectively target the angiogenic process could bring hope to millions of sufferers with cancer.

Punica granatum belonging to family Punicaceae is more commonly known as pomegranate (Salgado et al. 2006). ***Punica granatum*** is a large shrub which grows 12-16 feet, has many spiny branches with lance shaped glossy leaves. The bark of the tree turns gray as the tree ages. The flowers are large, red, white, or variegated having a tubular calyx that eventually becomes the fruit. Pomegranate is considered "A pharmacy unto itself" (Jurenka 2008). Pomegranate has many potential effects including bactericidal, antifungal, antiviral, immune modulation, vermifuge, stimulant, refrigerant, astringent, stomachic, styptic, laxative, diuretic and antihelminthic. It has also been widely used in treatment of cardiovascular diseases, diabetes, diarrhea, dysentery, asthma, bronchitis, cough, bleeding disorders, fever, inflammation, acquired immune deficiency syndrome, dyspepsia, ulcers, bruises, sores, mouth lesions, skin lesions, malaria, prostate cancer, atherosclerosis, hypertension, hyper lipidemia, denture stomatitis, male infertility, vaginitis, erectile dysfunction, Alzheimer's disease, obesity, and neonatal hypoxic-ischemic brain injury (Abdollahzdeh et al 2011; Jurenka 2008; Prakash et al. 2011). *Punica granatum* Linn roots are found to be rich in ellagitannins, including punicalin and punicalagin, numerous piperidine alkaloids (Tanaka et al. 1986; Neuhofer 1993). They are known for antihelmintic and vermifuge properties (Naqvi et al. 1991) and were found to be effective against melanoma in mice (Suresh et al. 2012). From the literature review, it was noted that fruit extract, peel extract (Dana et al. 2015) and pomegranate juice (Tibullo et al. 2016) possessed ant-angiogenic activity and it was noted that very limited study has been carried out in roots

of *Punica granatum.* Moreover, no scientific report is available regarding antiangiogenic and vasculoprotective potential of *Punica granatum* roots to the best of my knowledge. Based on this, the present study was planned to evaluate anti-angiogenic and vaculoprotective property of *Punica granatum* roots in chorioallantoic membrane model.

PLAN OF WORK

The present study was planned to evaluate the ant-angiogenic and vasculoprotective effect of *Punica granatum* root extract. The plan of work is

- Collect fresh roots of *Punica granatum* from a healthy and fresh well grown tree.
- To authenticate the roots of *Punica granatum.*
- To shade dry and to powder the roots to a coarse material.
- To extract the root powder using ethanol as solvent system by continuous hot extraction using soxhlet apparatus.
- To evaluate the effect of crude ethanol extract on angiogenesis in chorioallantoic membrane (CAM) model.
- Based on the activity of crude extract on vacularization in CAM model, the extract will be subjected to fractionation using solvents of increasing polarity.
- The fractions will be evaluated for the effect on angiogenesis in chorioallantoic membrane (CAM) model.
- The potent extract will be subjected to vasculoprotective role against glucose induced vascular changes.
- The results will be statistically analyzed for significance using student t test and one way ANOVA followed by Dunnett's test.

MATERIALS AND METHODS

The following instruments/apparatus were used for the present study

Table 5: Instrument and Apparatus were used

S.no	Instruments
1	Autoclave
2	BOD incubater
3	Laminar flow
4.	pH meter
5.	Digital balance
6.	Micropippete

Plant Material

The root material of *Punica granatum* Linn. of family Punicaceae were collected from Agricultural field, Pudupattu village, Villupuram district, Tamilnadu, India. The plant material was taxonomically indentified, confirmed and authenticated by ABS Botanical garden (AUT/ECP/109) was retained in our laboratory for further reference. The collected roots were shade dried and the dried materials were crushed to coarse powder with mechanical grinder. The powder was stored in an airtight container for extraction.

Extraction

About 186 gms of powdered root material of *Punica granatum* were extracted with 800 ml of ethanol in soxhlet apparatus for 72 hours. After extraction, the solvents were removed by distillation and evaporated to obtain crude extract of *Punica granatum* roots.

Phytochemical screening

The extract obtained was subjected to preliminary phytochemical screening (Kokate, 1994; Rosenthaler, 1930)

Detection of alkaloids

Individually the extracts were dissolved in dilute Hydrochloric acid and filtered. The filtered extract was subjected to detection of alkaloids.

Mayer's Test

Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). The formation of a yellow coloured precipitate indicates the presence of alkaloids.

Wagner's Test

Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). The formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's Test

Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). The formation of red precipitate indicates the presence of alkaloids.

Hager's Test

Filtrates were treated with Hager's reagent (saturated picric acid solution). The formation of yellow coloured precipitate indicates alkaloids.

Detection of carbohydrates

The extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Molisch's Test

Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. The formation of the violet ring at the junction indicates the presence of Carbohydrates.

Benedict's Test

Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Fehling's Test

Filtrates were hydrolysed with dil Hcl, neutralized with alkali and heated with Fehling's A & B solutions. The formation of red precipitate indicates the presence of reducing sugars.

Detection of glycosides

Individually the extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test

The extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Legal's Test

The extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. The formation of pink to blood red colour indicates the presence of cardiac glycosides.

Keller Killiani Test

Small portion from the extract was shaken with 1ml of Glacial acetic acid containing trace of ferric chloride. 1ml of Conc H_2So_4 was added carefully by the sides of the test tube. A blue colour in the acetic acid layer and red color at the junction of two liquids indicate the presence of glycosides.

Detection of saponins

Froth Test

Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Foam Test

0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of phytosterols

Salkowski's Test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

Libermann Burchard's test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

Detection of phenols

Ferric Chloride Test

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Detection of tannins

Gelatin test

To the extract 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins. 2ml of filtrate, few drops of lead acetate solution were added in a test tube. Formation of yellow precipitate indicates the presence of Tannins.

Detection of flavonoids

Alkaline Reagent Test

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate test

Extracts are treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Detection of proteins and aminoacids

Ninhydrin Test

To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

Xanthoproteic test

The extracts were treated with few drops of concentrated nitric acid. Formation of yellow colour indicates the presence of proteins.

Detection of Fixed oils and Fats

Oily Spot Test

One drop of extract was placed on filter paper and the solvent was evaporated. An oily stain of filter paper indicates the presence of fixed oil.

Quantitative estimation of bioactive compounds in crude ethanol extract of *Punica granatum* roots

Determination of phenolic content

Principle

This method strongly relies on the reduction of the mixture heteropolyphospho tungsates-molybdates by the phenolic compound which results in the formation of blue

coloured chromogen. The phenolic compounds react with folin-ciocalteu reagent only under basic conditions adjusted by sodium carbonate solution. Under basic conditions, it has been observed that the phenolic compounds undergoes dissociation to form a phenolate anion which reduces the folin-ciocalteu reagent ie the mixture of tungstates and molybdates rendering a blue coloured solution. The coloured intensity of the formed blue chromogen can be measured by the absorbance reading using spectrophotometic.

Procedure

The total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu method. It is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (FC reagent). This method, based on the Slinkard and Singleton, 1977 and the early work of Singleton & Rossi, 1965 is a colorimetric oxidation/reduction method for phenolic compounds. A blue colour which is the product of metal oxidation, that exhibits a broad light absorption with a maximum at 764 nm. The intensity of light absorption is proportional to the concentration of phenols. 20 µL of the diluted sample was added to 100 µL of Folin-Ciocalteu reagent. After 8 min, 300 µL of saturated sodium carbonate solution (25%) was added. The absorbance was measured at 764 nm. The calibration curve was prepared with gallic acid solutions ranging from 10 to 1000 µg/ml, and the results are given as gallic acid equivalents (GAE).

Determination of total flavonoids

Principle

This method is based on the formation of the flavonoid aluminium complex which has an absorptivity maximum at 415nm. The principle of the method used for the determination of flavonoid content consist in the fact that $AlCl_2$ forms stable acid complexes with carbonyl group at C_4 and hydroxyl group at C_3 and C_5 in flavanols and flavones. In addition it forms acid labile complexes with hydroxyl in the orthoposition in A or B rings of flavonoids.

Procedure

Flavonoid content was measured using aluminium chloride colorimetric method. Various concentrations of extract were mixed with 0.1 ml of 10 % aluminium chloride (w/v), 0.1 ml of 1 M potassium acetate and 2.8 ml distilled water. The mixture was allowed to stand at room temperature for 30 minutes. The absorbance of reaction mixture was measured at 415 nm. Results are expressed as mg/g quercetin equivalent (Chang, 2002).

Evaluation of total antioxidant capacity by Phosphomolybdenum method

Principle

Antioxidant present in the sample reduce the MO(VI) to MO (V), which then react with phosphate group of sodium phosphate to form green coloured MO(V)- phosphate complex (phosphomolybdenum complex) in an acidic medium. This complex is then spectrophotometrically measured at 695nm. This reaction is highly time dependent.

Procedure

The antioxidant activities extract was evaluated by the phosphomolybdenum method according to the procedure described by Prieto *et al*., 1999. The assay is based on the reduction of Mo (VI) – Mo (V) by the extract. The subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 ml different concentrations of extract (10μg/ml to 200 μg/ml) were mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). 0.3 mL of methanol was used as blank in place of extracts. The tubes were incubated in a boiling water bath at 95°C for 90 min. The absorbance of the solution was measured at 695 nm after cooling to room temperature. The antioxidant capacity of each sample was expressed as ascorbic acid equivalent.

Determination of free radical scavenging activity

DPPH radical scavenging assay

Principle

DPPH is a stable nitrogen centered free radical which can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Any molecule that can donate an electron or hydrogen to DPPH can react with it thereby bleach the DPPH absorption. DPPH is a purple colour dye having absorbtion maximum of 517 nm and upon reaction with hydrogen donar, the purple colour fades or disappears due to conversion to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance. Substances which are able to perform the reaction can be considered as antioxidant and therefore radical scavengers.

Procedure

Free radical scavenging activity was determined spectrophotometrically using the method of Blois, 1958. This method is based on the measurement of the reducing ability of antioxidants toward the DPPH radical. Briefly, 100 µl of various concentrations of the root extract (1.95 µg/ml to 1000 µg/ml) in methanol were added to 10 ml of a methanol solution of DPPH ($1.01.10^{-2}$ M). The mixture was allowed to stand at room temperature for 30 min in the dark after a vigorous shake. The absorbance was measured at 517 nm. The control mixture consists of 100 µl of methanol and 10 ml of DPPH solution. The scavenging activity on the DPPH radical was calculated as inhibition percentage using the following equation:

$$\% \text{ Inhibition} = [(AB - AS)/AB] \times 100$$

where AB is the absorbance of the control reaction (containing all reagents except the test compound), and AS is the absorbance of the test compound. Ascorbic acid was used as reference standard. The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration.

Ferric reducing antioxidant capacity

Principle

The reducing capacity of the compound can be measured by direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. Addition of free Fe^{3+} to the reduced product leads to formation of intense Perl's prussion blue complex, $Fe_4[(CN)_6]_3$, which has a strong absorbance at 700 nm. An increase in absorbance of the reaction mixture would indicate an increase in the reducing capacity due to an increase in the formation of the complex. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending upon the reducing power of antioxidant samples.

Procedure

According to the method described by Oyaizu, 1986 the reducing powers of extract were determined. Different concentrations of root extract (1.95 µg/ml to 1000 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). After incubation at 50°C for 20 min 2.5 ml of trichloroacetic acid (10%) was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid was used as reference standard.

Effect of crude ethanol extract of *Punica granatum* roots on vascularisation in chick chorioallantoic membrane (CAM) model

Fertile chicken eggs (*Gallus domesticus*) were obtained from poultry farm in Namakkal district and were kept in a humidified incubator at 37°C in horizontal position. The eggs were rotated three times a day to ensure uniform embryo development. On day 3, eggs were swabbed with 70 % alcohol. Under a laminar flow hood, 2–3 ml of albumin was

aspirated from eggs with a syringe without needle through a small hole drilled at the narrow end of the eggs, allowing the developing CAM to detach from the shell membrane. The holes were then closed with plaster. The upper surface of the shell was then cut to make a window with a sterile forceps under laminar air flow. This window served as a portal of access for the CAM. The window was then closed with a cellophane tape and the eggs were returned to the incubator. On day 8, the window was opened and the CAM was accessed and photographed and the eggs were grouped into four groups of six each. Absorbable gelatin sponge soaked in 100 µg of crude ethanol extract of *Punica granatum* in PBS was placed directly using surgical forceps on CAM surface to one group of eggs. To another group of eggs, absorbable gelatin sponge soaked in 200 µg of crude ethanol extract of *Punica granatum* in PBS was placed directly using surgical forceps on CAM surface. A group of eggs served as solvent control in which absorbable gelatin sponge soaked in 0.5 µl of PBS was placed on CAM surface and a group served as normal egg without treatment to access angiogenesis. All the eggs were then returned to the incubator. The experiments were performed between days 8 and 12 of incubation period because, it is generally accepted that days 8 to 10 are strongly angiogenic. On day 12 of incubation, the CAM was inspected for microvessel density. The blood vessels were counted including the vessels radially converging towards the center in an area of 80 mm×80 mm around the sponge using Photoshop Elliptical Marquee tool (Nguyen *et al*., 1994; Tufan and Satiroglu-Tufan, 2005; Koneri *et al*., 2014)..

The percentage inhibition was calculated using the following equation

Percentage inhibition = Vessel number of CAM treated by normal saline - Vessel number of CAM treated by extract / Vessel number of CAM treated by normal saline X 100.

Based on the results obtained from the effect of crude extract of *Punica granatum* roots on vascularisation in chick chorioallantoic membrane (CAM) model, the extract is subjected to fractionation.

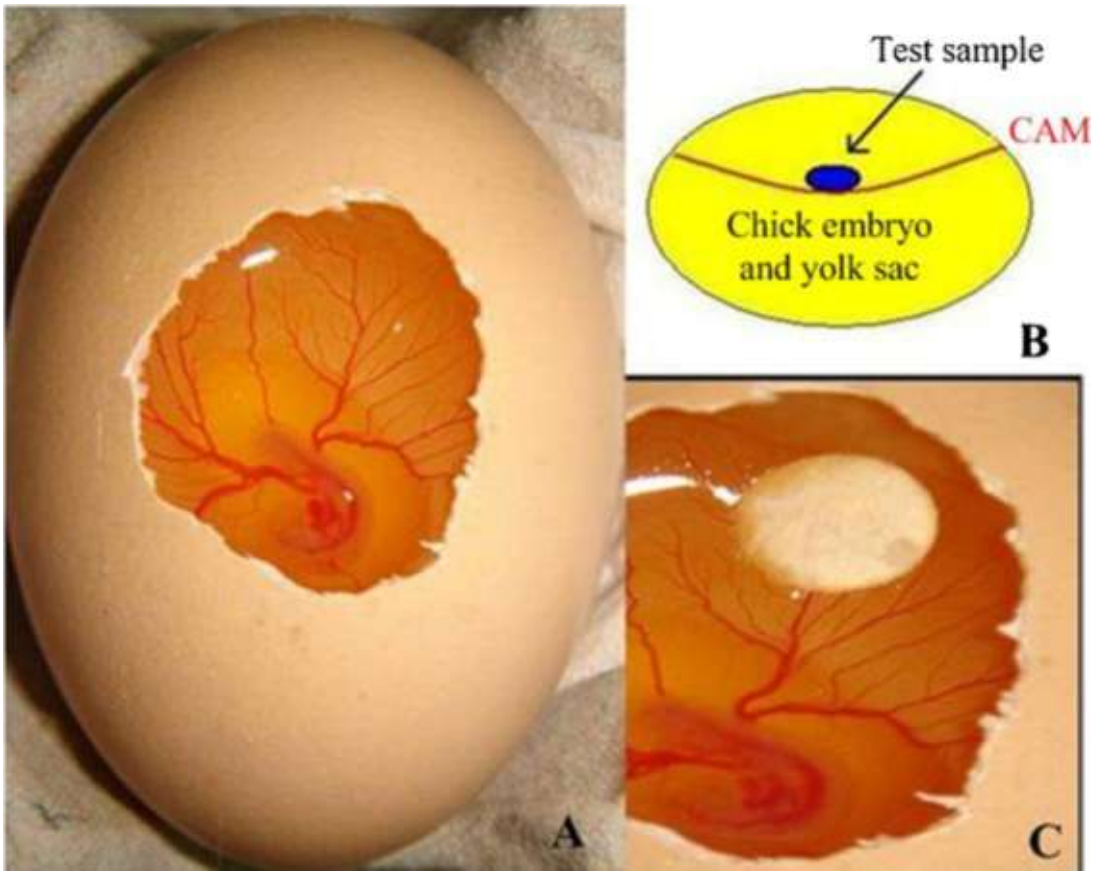


Figure. 18 Vascularisation in chick chorioallantoic membrane (CAM) Model.

A. window made in the egg shell of a 3-day-old chick embryo

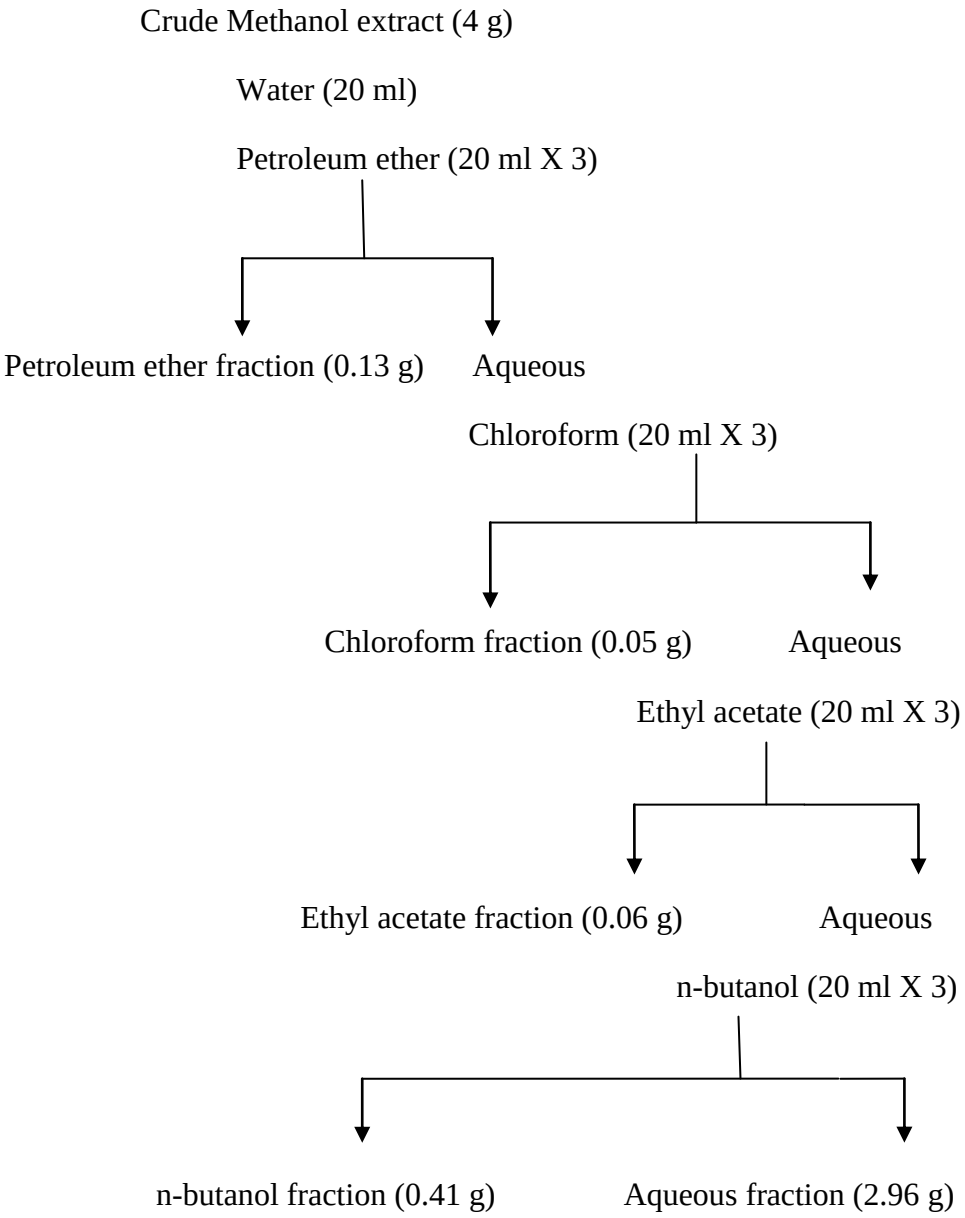
B Diagram of the technique

C. filter paper discs/gelatin sponge with the test substances placed manually

by surgical forceps onto the CAM of a 8-day-old chick embryo

Fractionation of crude extract of *Punica granatum* roots.

About 4 g of the dried ethanol extract was dissolved in 20 ml water and was successively partitioned with petroleum ether, chloroform, ethyl acetate and n-butanol. The percentage yield of the resulting extracts were calculated and shown in table --. Crude extract and fractions were encoded as: CE – Crude ethanol Extract; PEF- Petroleum Ether Fraction; CF- Chloroform Fraction; EAF- Ethyl Acetate Fraction; BF- n-butanol fraction; AF- Aqueous Fraction remaining after fractionation. All extract fractions were freeze dried and stored at 4°C (Magaji et al., 2007).



Fractionation scheme of *Punica granatum* roots

Angiogenesis inhibitory effects of various fractions from crude ethanol extract of *Punica granatum* roots

Thirty Fertile chicken eggs (*Gallus domesticus*) were used for the evaluation of angiogenesis inhibitory effects of various fractions from crude ethanol extract of *Punica granatum*. The eggs were rotated three times a day to ensure uniform embryo development. On day 3, eggs were swabbed with 70 % alcohol. Under a laminar flow hood, 2–3 ml of

albumin was aspirated from eggs with a syringe without needle through a small hole drilled at the narrow end of the eggs, allowing the developing CAM to detach from the shell membrane. The holes were then closed with plaster. The upper surface of the shell was then cut to make a window with a sterile forceps under laminar air flow. This window served as a portal of access for the CAM. The window was then closed with a cellophane tape and the eggs were returned to the incubator. On day 8, the window was opened and the CAM was accessed and photographed and the eggs were grouped into five groups of six each for evaluation of five fractions. Absorbable gelatin sponges soaked in 100 µg of various fractions were placed directly using surgical forceps on CAM surface to respective group of eggs. All the eggs were then returned to the incubator. On day 12 of incubation, the CAM was inspected for microvessel density. The vessels were counted and the percentage inhibition was calculated (Nguyen *et al*., 1994; Tufan and Satiroglu-Tufan, 2005; Koneri *et al*., 2014).

Effect of Ethyl acetate fraction of *Punica granatum* on hyperglycemia induced vascular changes in chick chorioallantoic membrane model

Fertile chicken eggs (*Gallus domesticus*) were obtained from poultry farm and were incubated at 38° C in a humidified environment in horizontal position. The eggs were rotated three times a day to ensure uniform embryo development. On day 3, eggs were swabbed with 70 % alcohol. Under a laminar flow hood, 2 ml of albumin was aspirated from eggs with a syringe through a small hole drilled at the narrow end of the eggs, allowing the developing CAM to detach from the shell membrane. The holes were then closed with plaster. The upper surface of the shell was then cut to make a window with a sterile forceps under laminar air flow. The window was then closed with a sterile transparent tape and the eggs were returned to the incubator. By day 8 of post incubation, thirty eggs were selected for experiment and divided into five groups of six each. Group I vehicle control eggs received a single intravitellus injection of 1 ml sterile water. In group II eggs, hyperglycemia was induced by a

single intravitellus injection of 5 mg glucose/g whole egg (Glucose stock solution: 30 % w/v in water). Group III eggs received 100 µg EAF-PG dissolved in glucose solution (5 mg glucose/g whole egg). On day 12 of incubation, blood glucose level was measured in the samples taken from the CAM vessel using Accu-check glucometer and the blood vessels were inspected for any vascular change/abnormalities in the respective groups (Larger et al. 2004; Di Marco et al. 2008). The CAM was inspected and photographed using a digital camera with a magnification of 20 X. The blood vessels were counted including the vessels radially converging towards the center in an area of 80 mm×80 mm around the sponge using Photoshop Elliptical Marquee tool.

Statistical analysis

The data's were expressed as mean ± standard error of the mean (SEM). For paired comparisons, student's t-test analyses were performed. Different groups were assessed by one-way analysis of variance (ANOVA) for multiple comparisons followed by dunnett's test (Graphpad software Inc, La Jolla, CA. Trial version 5). The criterion for statistical significance was set at $P < 0.05$.

Table. 6 Percentage yield of ethanol extract of *Punica granatum* root (EEPG)

S.NO	Extract	Percentage Yield (w/w)
1	Crude ethanol extract of *Punica granatum* root (EEPG)	42.86 %

186 g of dried powder of *Punica granatum* root yielded 79.70 g of crude ethanol extract (EEPG) which was about 42.86 % of the root powder subjected to extraction.

Table.7 Qualitative phytochemical screening of EEPG

Phytochemicals	Test	EEPG
Alkaloids	a. Mayer's test b. Dragendroff's c. Hager's test d. Wagner's test	+ + - +
Phytosterols And Triterpenes	a. Leiberman-Burchard b. Salkowaski test	- -
Flavonoids	a. Alkaline reagent test b. Lead acetate test	+ +
Saponins	a. Foam test	+
Proteins and Aminoacids	a. Ninhydrin test b. Xanthoproteic test	- +
Phenolics	a. Ferric chloride test	+
Tannins	a. Gelatin test	+
Carbohydrate	a. Molisch test b. Fehling's test c. Benedict's test	+ + +
Gylcosides	a. Modified Borntrager's test b. Legal's test c. Keller-Killiani test	+ - +

Presence (+) Negative (-)

Table.8 Total Phenolic content (TPC), Total Flavonoid content (TFC) and Total antioxidant capacity (TAC) of EEPG

Extract	TPC (µg of GAE/mg of extract)	TFC (µg of quercetin/mg of extract)	TAC (µg of quercetin /mg of extract)
EEPG	237.60	247.60	441.01

Total Phenolic content (TPC) is expressed as milligram of gallic acid equivalent per gram of sample. Total tannin content (TTC) is expressed as milligrams of tannic acid equivalents per gram of dry extract. Total Flavonoid content (TFC) is expressed as milligrams of quercetin equivalents per gram of dry extract. Total antioxidant capacity is expressed as milligrams of quercetin equivalents per gram of dry extract.

Table.9 Free radical scavenging activity of EEPG

Extract	DPPH radical scavenging (µg/mL) IC_{50}	Nitric oxide scavenging activity (µg/mL) IC_{50}
EEPG	114	389
Standard	15.52	12

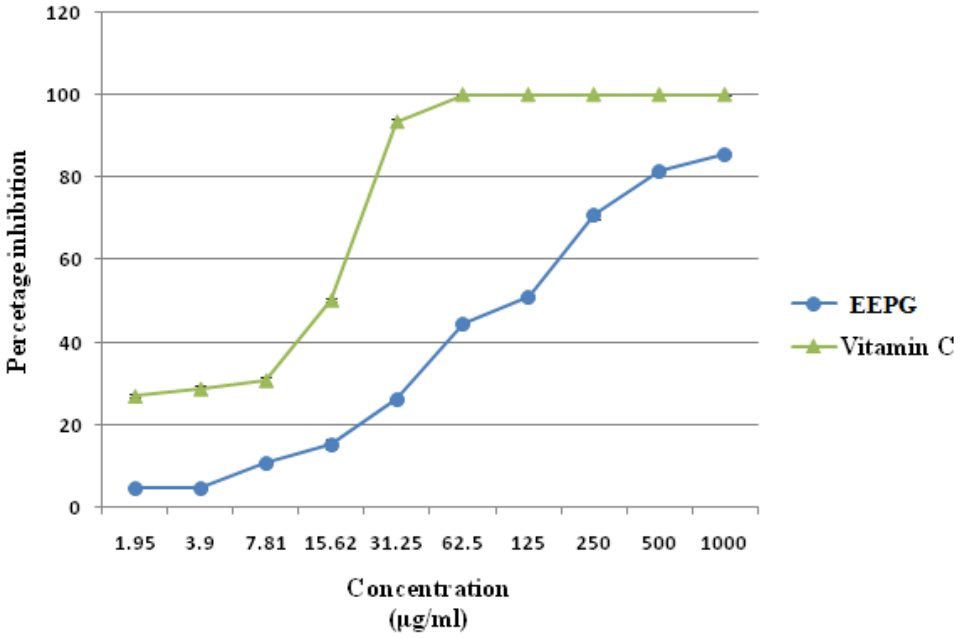


Figure.19 DPPH SCAVENGING ACTIVITY

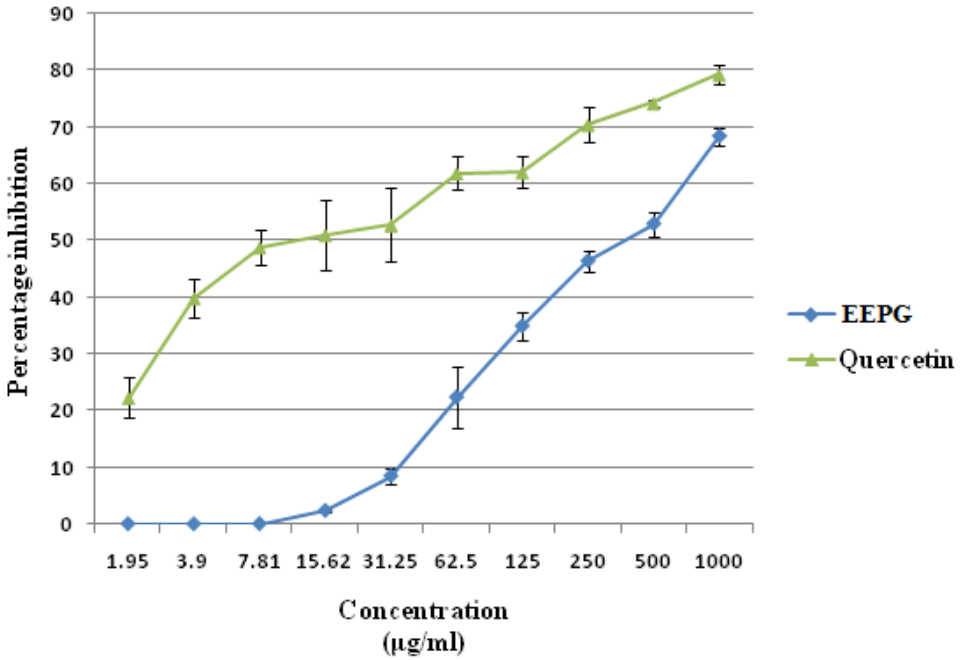


Figure.20 NITRIC OXIDE SCAVENGING ACTIVITY

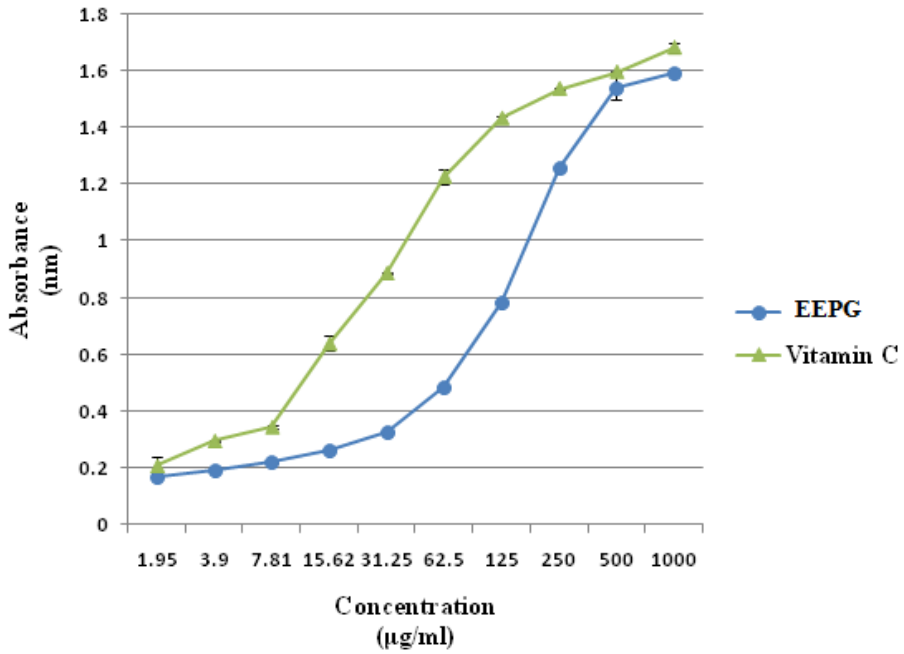


Figure.21 FERRIC REDUCING ANTIOXIDANT CAPACITY

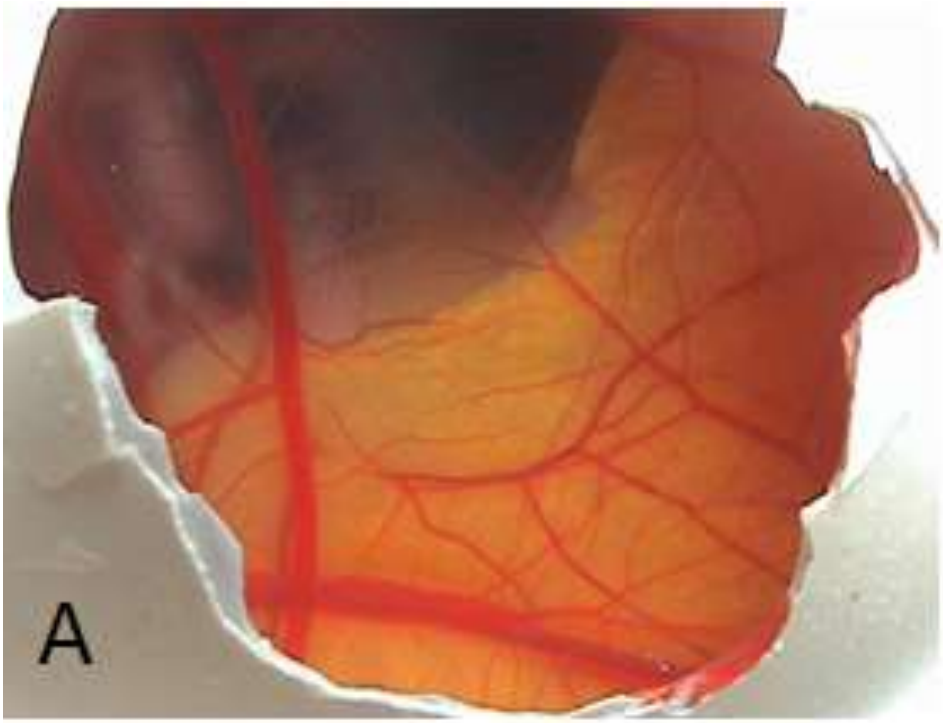


Figure.22 (a) Normal control

Figure.22 (b) PBS control

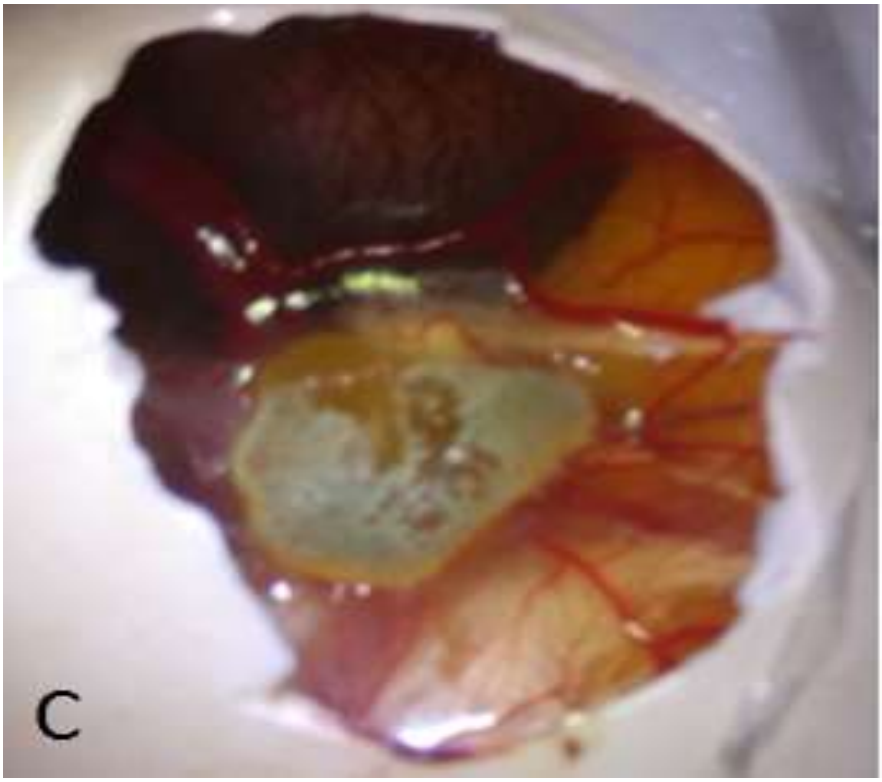


Figure 22 (c) EEPG (100 µg)

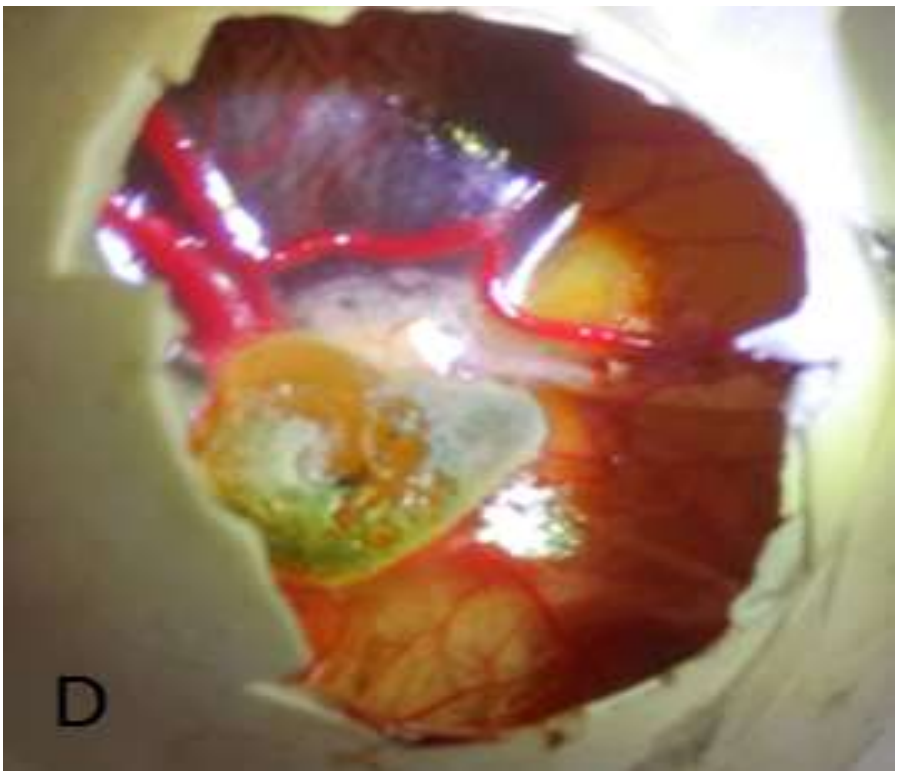


Figure 22 (d) EEPG (100 µg)

Figure.22 Effect of EEPG extract on vascularisation in chick chorioallantoic membrane (CAM) mode

Table.10 Effect of EEPG extract on vascularisation in chick chorioallantoic membrane (CAM) model

Treatment	Average Vessel Number
Normal Egg	59.67 ± 1.52
PBS Control	57.83 ± 1.16[ns a]
EEPG (100 µg)	14.67 ± 0.66[*** b]

All values are expressed as mean ± S.E.M, n=6 in each group.

[a] Values are significantly different from normal control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001. (Student t test analysis)

[b]Values are significantly different from PBS control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001. (Student t test analysis)

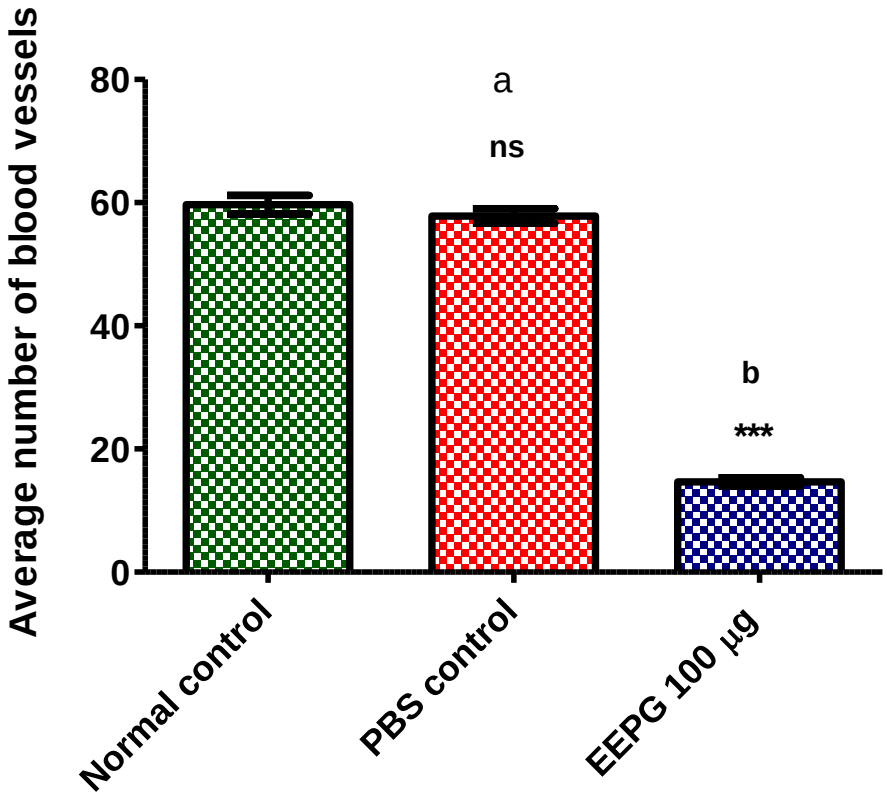


Figure.23 Effect of EEPG extract on vascularisation in chick chorioallantoic membrane (CAM) model

All values are expressed as mean ± S.E.M, n=6 in each group.

[a] Values are significantly different from normal control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001. (Student t test analysis)

[b]Values are significantly different from PBS control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001. (Student t test analysis)

Fractionation of crude extract

Table.11 The percentage yield of various fractions of *Punica granatum* root extract

S.No	Extract/Fractions	Percentage Yield (w/w)
1	Petroleum ether Fraction (PEF-PG)	3.25 %
2	Chloroform Fraction (CF-PG)	1.25 %
3	Ethyl acetate Fraction (EAF-PG)	1.5 %
4	n-Butanol Fraction (BF-PG)	10.25 %
5	Aqueous Fraction (AF-PG)	74 %

Fractionation of 4 g methanol extract using petroleum ether as solvent yielded 0.13 g which was about 3.25 % of the extract subjected to fractionation. Successive fractionation with chloroform yielded 0.05 g, which was about 1.25 % of the extract subjected to fractionation. Successive fractionation with ethyl acetate yielded 0.06 g, which was about 1.5 % of the extract subjected to fractionation. Successive fractionation with n-butanol yielded 0.41 g, which was about 10.25 % of the extract subjected to fractionation. The remaining aqueous fraction after fractionation yielded 2.96 g, which was about 74 % of the extract subjected to fractionation. All extract fractions were freeze dried and stored at 4 °C.

Figure 24 (a)

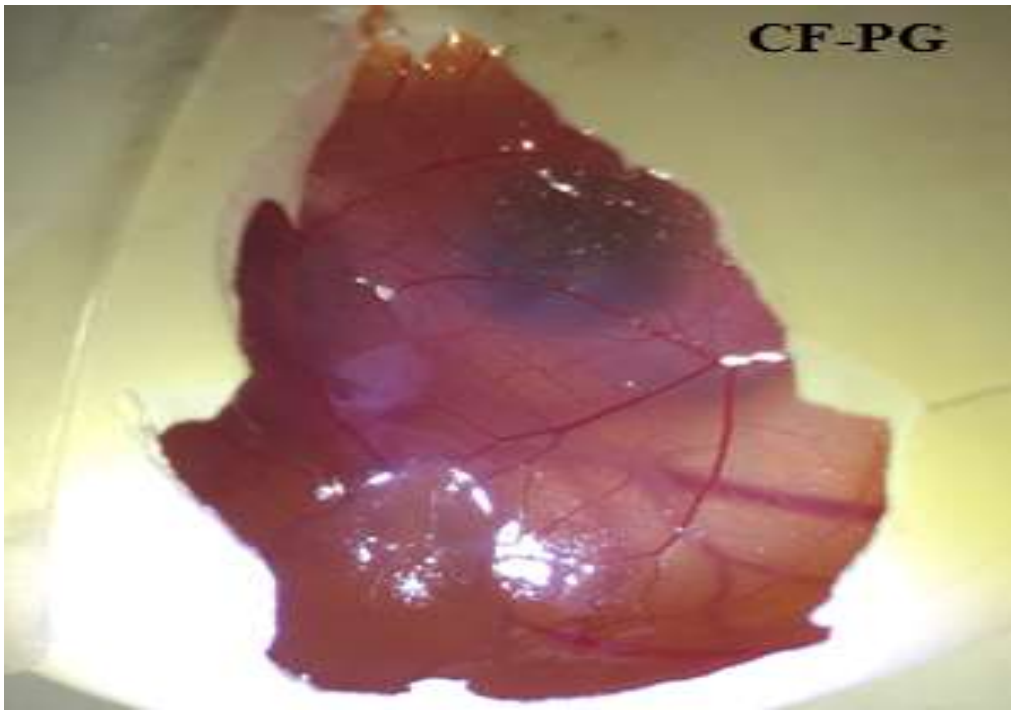


Figure 24 (b)

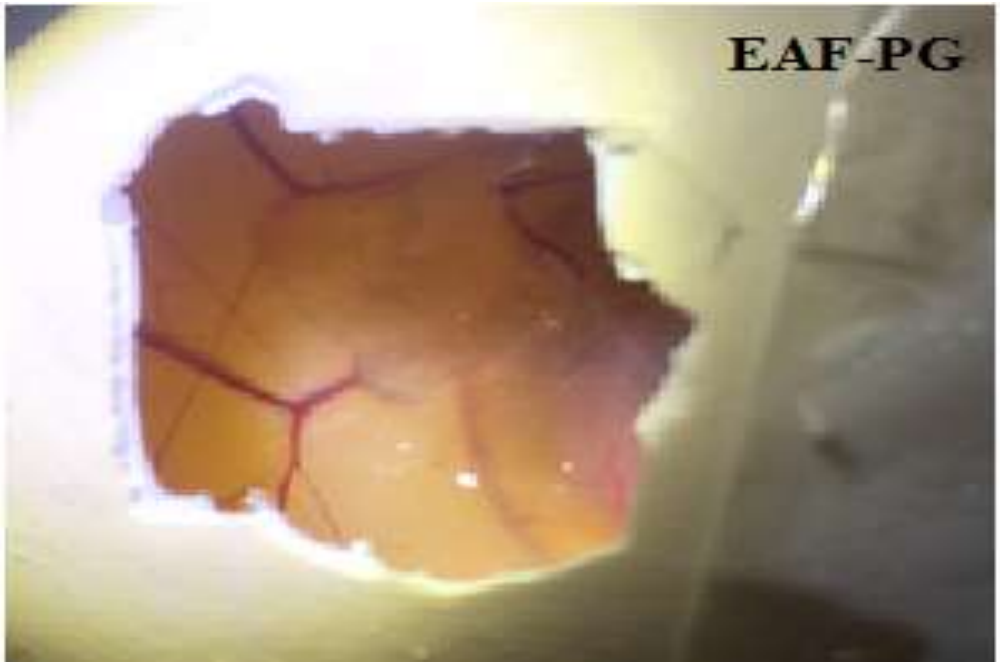


Figure 24(c)

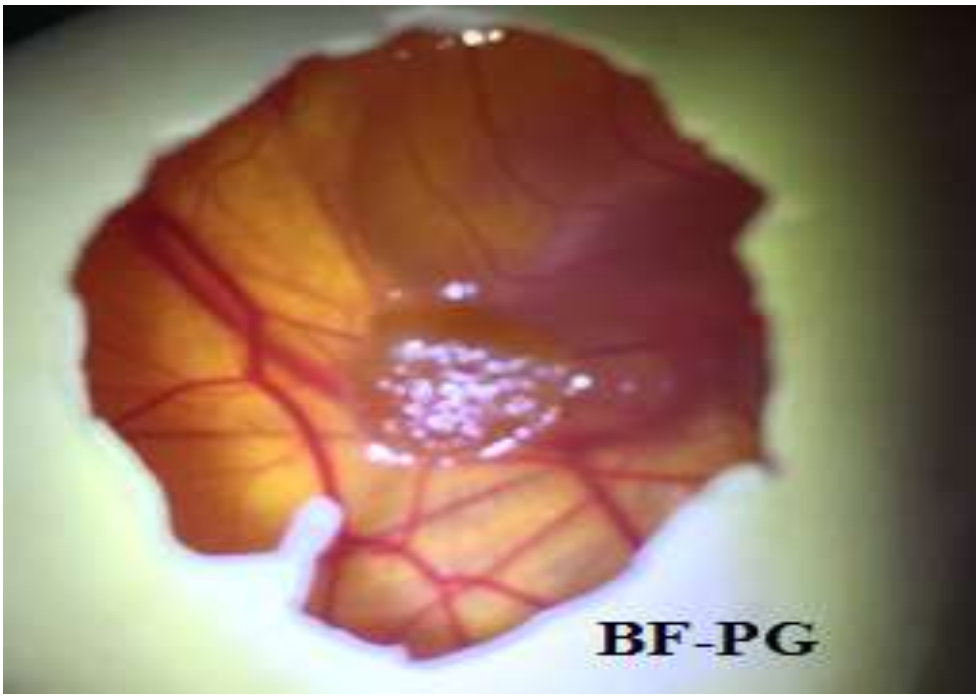


Figure 24(d)

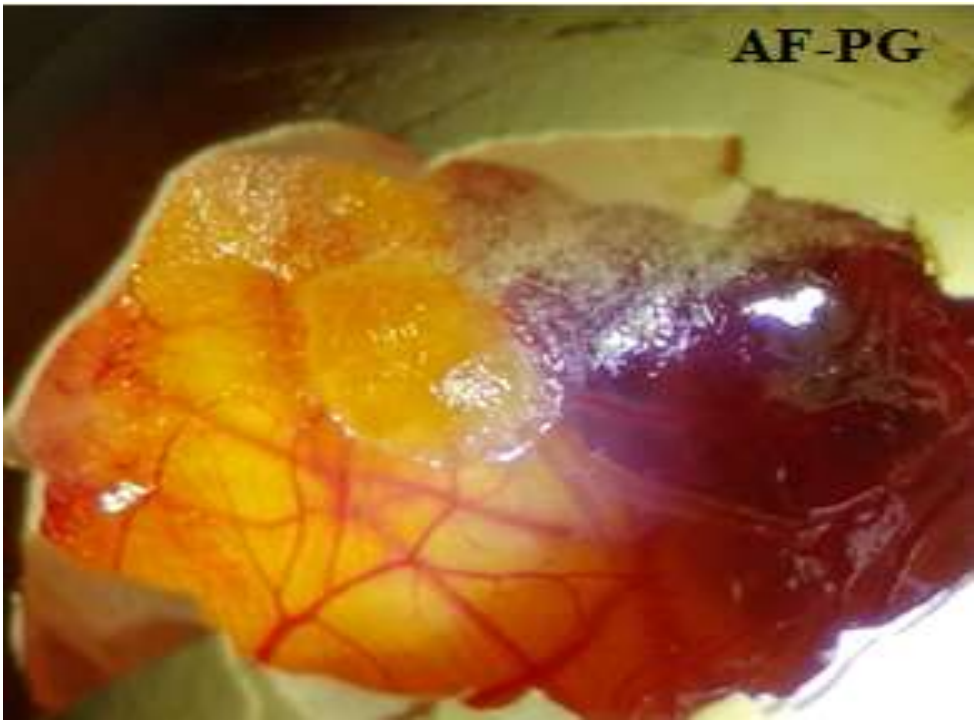


Figure 24 (e)

Figure. 24 Effect of various fractions from EEPG on vascularization in chick chorioallantoic membrane (CAM) model

Table.12 Effect of various fractions from EEPG on vascularisation in chick chorioallantoic membrane (CAM) model

Treatment	Average Vessel Number
Normal Egg	59.67 ± 1.52
PBS Control	57.83 ± 1.16$^{ns\ a}$
PEF-PG	47.33 ± 1.25$^{***\ b}$
CF-PG	29.00 ± 0.96$^{***\ b}$
EAF-PG	20.33 ± 0.76$^{***\ b}$
BF-PG	32.17 ± 0.65$^{***\ b}$
AF-PG	28.67 ± 0.66$^{***\ b}$

All values are expressed as mean ± S.E.M, n=6 in each group.

[a] Values are significantly different from normal control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001. (Student t test analysis)

[b] Values are significantly different from PBS control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001. (One -way ANOVA followed by Dunnett's test was used to compare experimental groups).

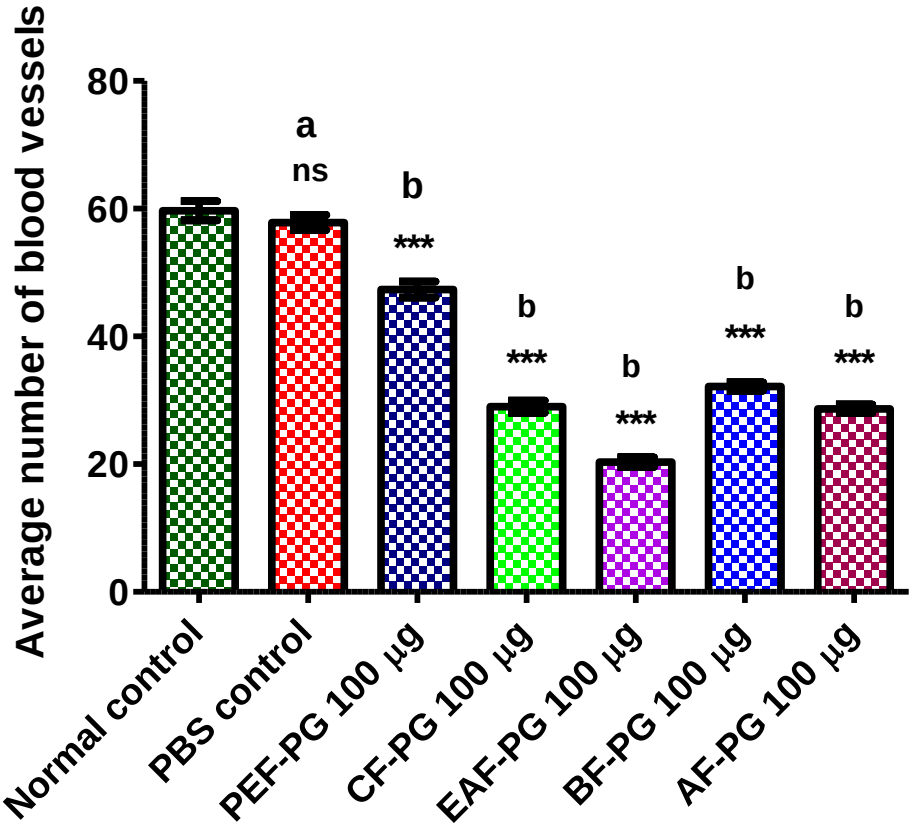


Figure.25 Effect of various fractions from EEPG on vascularisation in chick chorioallantoic membrane (CAM) model

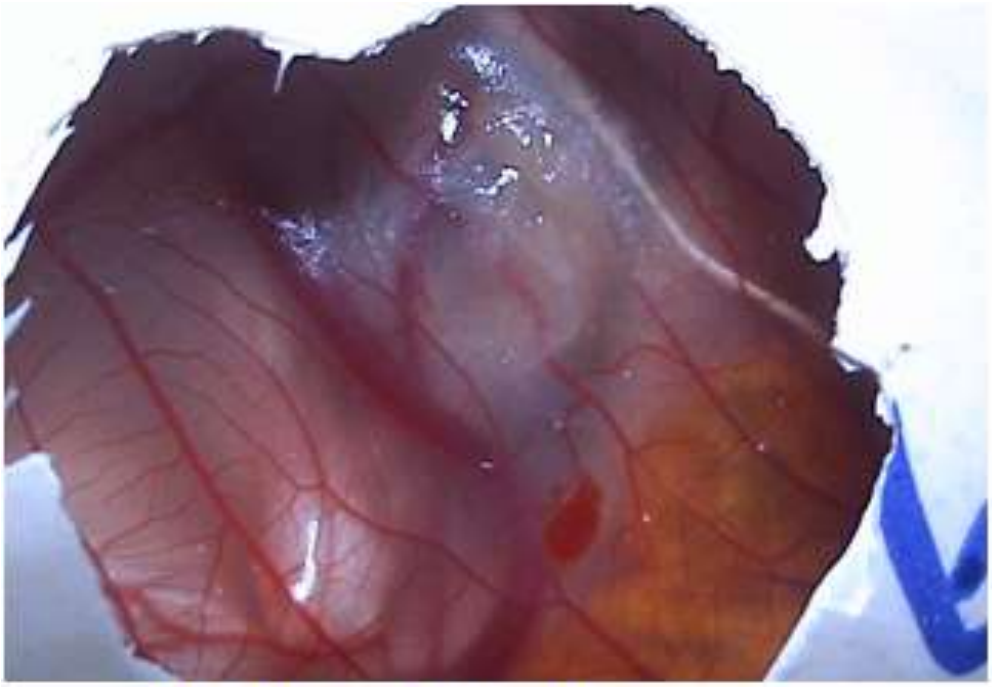
All values are expressed as mean ± S.E.M, n=6 in each group.

[a] Values are significantly different from normal control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001. (Student t test analysis)

[b] Values are significantly different from PBS control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001. (One -way ANOVA followed by Dunnett's test was used to compare experimental groups).

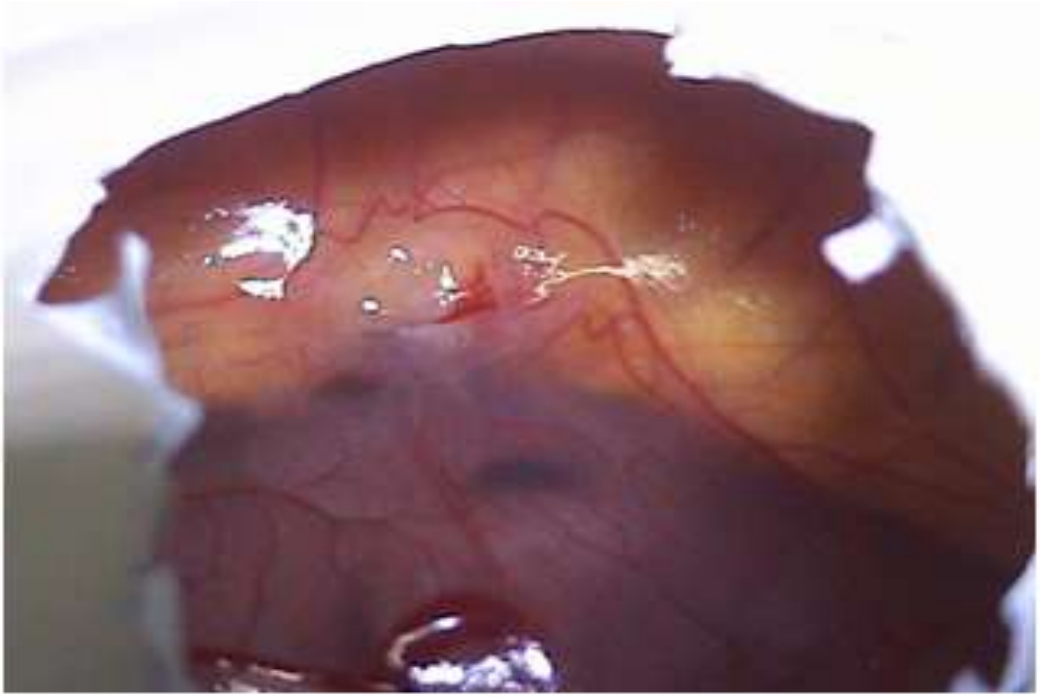
VEHICLE TREATED (STERILE WATER)

Figure 26 (a)



GLUCOSE INDUCED

Figure 26 (b) **<u>Hyperglycaemia induced (5 mg glucose/g whole egg)</u>**



GLUCOSE INDUCED

Hyperglycaemia induced (5 mg glucose/g whole egg)

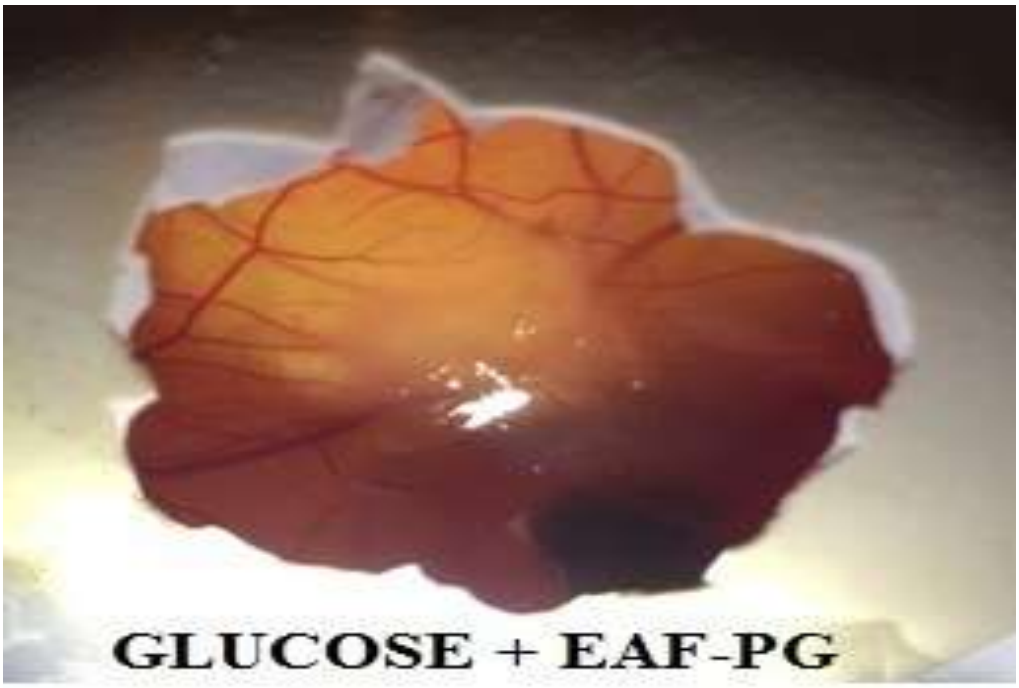


Figure 26 (c) Hyperglycemia induced (5 mg glucose/g whole egg) + EAF-PG

Figure.26 Effect of Ethyl acetate fraction of *Punica granatum* on hyperglycemia induced vascular changes in chick chorioallantoic membrane model.

Table.13 Effect of EAF-PG extract on vascularisation in hyperglycemia induced chick chorioallantoic membrane model

Treatment	Average Vessel Number
Sterile water	31.00 ± 0.96
Glucose 5 mg/g of whole egg	54.00 ± 1.23$^{***\ a}$
Glucose + EAF-PG 100 µg	42.33 ± 1.05$^{**\ b}$

All values are expressed as mean ± S.E.M, n=6 in each group.

[a] Values are significantly different from sterile water (vehicle) group; ns-non significant;

*P < 0.05; **P < 0.01; ***P < 0.001. (Student t test analysis)

[b]Values are significantly different from Glucose induced group; ns-non significant; *P < 0.05;

**P < 0.01; ***P < 0.001. (Student t test analysis)

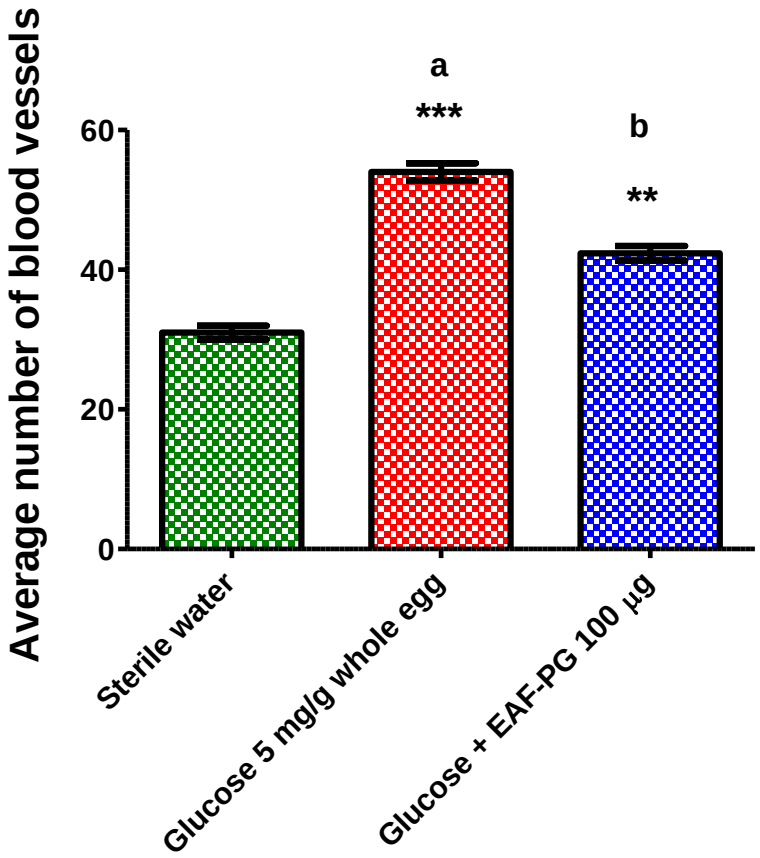


Figure.27 Effect of EAF-PG extract on vascularisation in hyperglycemia induced chick chorioallantoic membrane model

All values are expressed as mean ± S.E.M, n=6 in each group.

[a] Values are significantly different from sterile water (vehicle) group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001. (Student t test analysis)

[b]Values are significantly different from Glucose induced group; ns-non significant; *P < 0.05;

**P < 0.01; ***P < 0.001. (Student t test analysis)

Table.14 Effect of EAF-PG extract on blood glucose level in hyperglycemia induced CAM

Group	Blood glucose (mg/dl)
Sterile water	73.50 ± 1.25
Glucose 5 mg/g of whole egg	304.8 ± 2.85***a
Glucose + EAF-PG 100 µg	117.0 ± 2.63***b

All values are expressed as mean ± S.E.M, n=6 in each group.

[a] values are significantly different from group II Vehicle control ; ns – non-significant; P values: * p< 0.05, ** p< 0.01, *** p< 0.001 (Student t. test analysis).

[b] values are significantly different from group III glucose induced, ns- non significant, P values: * p<0.05, ** p< 0.01, *** p< 0.001 (one way ANOVA followed by Dunnett's test).

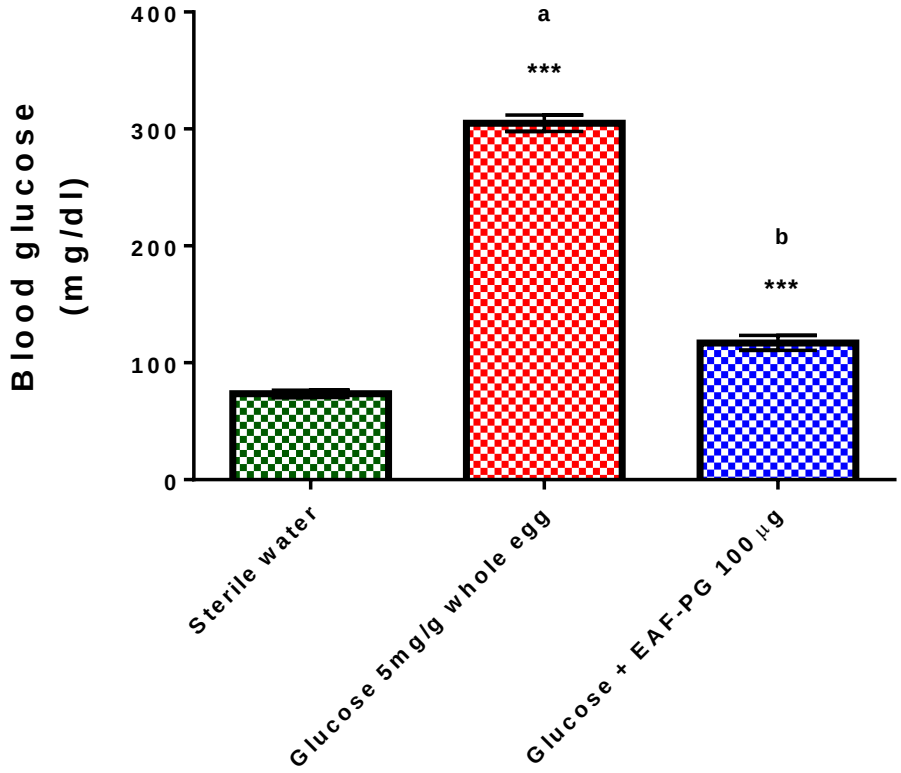


Fig. 28 Effect of EAF-PG extract on blood glucose level in hyperglycemia induced CAM

All values are expressed as mean ± S.E.M, n=6 in each group.

[a] values are significantly different from group II Vehicle control ; ns – non-significant; P values: * p□ 0.05, ** p□ 0.01, *** p□ 0.001 (Student t. test analysis).

[b] values are significantly different from group III glucose induced, ns- non significant, P values: * p□ 0.05, ** p□ 0.01, *** p□ 0.001 (one way ANOVA followed by Dunnett's test).

Percentage yield

The percentage yield of ethanol extract of *Punica granatum* root (EEPG) is shown in table 6**.** 186 g of dried leaf powder yielded 79.70 g of crude ethanol extract (CME-OT), which was about 42.86 % of the root powder subjected to extraction.

Phytochemical examination

Qualitative phytochemical screening of EEPG is shown in table.7. The phytochemical examination of EEPG revealed the presence of alkaloids, glycosides, flavonoids, tannins, saponins and carbohydrates.

Quantitative estimation of bioactive compounds

Total Phenolic content

The quantitative estimation of bioactive compounds in EEPG is shown in table. 8. The total phenolic content in EEPG was determined spectrophotometrically using folin-ciocalteu method and the results are expressed as gallic acid equivalents (GAE).

The total phenolic content in EEPG was found be 237.60 µg of GAE equivalent/mg of extract.

Total Flavonoid content

The total flavonoid content was determined by aluminium chloride calorimetric method and the results were expressed as quercetin equivalent. The total flavonoid content in EEPG was found be 247.60 µg of quercetin equivalent/mg of extract (Table.8).

Total antioxidant capacity

The total antioxidant capacity of EEPG was evaluated by phosphomolybdinum method and the results were expressed as quercetin equivalent. EEPG roots showed 441.01 µg of quercetin equivalent/mg of extract antioxidant activity (Table.8).

Free radical scavenging

The free radical scavenging activity of EEPG were investigated using DPPH scavenging activity, Nitric oxide scavenging activity and ferric reducing assay.

DPPH scavenging activity

The DPPH radical scavenging activity of EEPG are shown in table.9 and figure.19. A dose dependent inhibition of DPPH activity was observed with crude extract. The IC_{50} concentration of EEPG which caused inhibition of DPPH radical was 114 µg/ml.

As lower IC_{50} values indicate higher radical scavenging activity, DPPH showed a potent DPPH radical scavenging activity.

Nitric oxide scavenging activity

The nitric oxide scavenging activity of EEPG is shown in table.9 and figure 20. A dose dependent inhibition of nitric oxide formation was observed with crude extract. The IC_{50} concentration of EEPG which caused inhibition of nitric oxide radical was 389 µg/ml and the

Ferric reducing power assay

The ferric reducing power of EEPG are shown in figure. 21. It was observed that, EEPG showed a concentration dependent increase in reducing capacity.

Effect of EEPG root on vascularisation in chick chorioallantoic membrane (CAM) model

The effect of EEPG on vascularisation in chick chorioallantoic membrane (CAM) model is shown in table.10 and figure.22- figure.23. It was observed that, the microvessel density was significantly ($p<0.001$) decreased on day 12 of inspection in group of fertile chicken eggs incubated at 37°C in presence of EEPG (100 µg) placed on CAM surface on 8th day of incubation compared to PBS solvent control group of eggs. The average number of vessels observed on CAM surface in eggs incubated with PBS solvent control was found to be 57.83 ± 1.16. Whereas in eggs treated with EEPG, the number of vessels were reduced to 14.67 ± 0.66 ($p<0.001$).

Based on these results, EEPG extract was subjected for fractionation to evaluate the effect of fractions on angiogenesis in chorioallantoic membrane.

Percentage yield of various fractions of CME-PA

The Percentage yield of various fractions of EEPG is shown in table.11. Fractionation of 4 g methanol extract using petroleum ether as solvent yielded 0.13 g which was about 3.25 % of the extract subjected to fractionation. Successive fractionation with chloroform yielded 0.05 g, which was about 1.25 % of the extract subjected to fractionation. Successive fractionation with ethyl acetate yielded 0.06 g, which was about 1.5 % of the extract subjected to fractionation. Successive fractionation with n-butanol yielded 0.41 g, which was about 10.25 % of the extract subjected to fractionation. The remaining aqueous fraction after fractionation yielded 2.96 g, which was about 74 % of the extract subjected to fractionation. All extract fractions were freeze dried and stored at 4 °C.

Angiogenesis inhibitory effects of various fractions from EEPG in chick chorioallantoic membrane (CAM) model

The effect of various fractions from EEPG in chick chorioallantoic membrane (CAM) model is shown in table 12, figure 24 and 25. On day 12 of inspection, it was observed that the group of eggs treated with PEF-PG showed 47.33 ± 1.25 average number of vessels on CAM. The eggs treated with CF-PG showed 29.00 ± 0.96 average number of vessels on CAM. The eggs treated with EAF-PG showed 20.33 ± 0.76 average number of vessels on CAM. The eggs treated with BF-PG showed 32.17 ± 0.65 average number of vessels on CAM and the average number of blood vessels found in group of eggs treated with AF-PG showed 28.67 ± 0.66. Based on the angiogenesis inhibitory effect of fractions from EEPG on vascularisation in chick chorioallantoic membrane (CAM) model, the ethyl acetate fraction which showed a significant inhibitory effect was selected to study the effect on glucose induced vascular changes in CAM model.

The morphological changes in vessels were examined in CAM. There is no incidence of morphological changes in group I normal vehicle treated eggs. In hyperglycaemia induced group II eggs, microvascular abnormalities including vascular leakage with haemorrhagic spots (microaneurysms), proliferation of new vessels (neovascularization), superficial white lesions were observed. The incidence of vascular defects was 100 % in group II glucose induced eggs. Group III hyperglycaemia induced eggs treated with 100 µg EAF-PG showed normal vascular architecture with no incidence of vascular abnormality (Fig.26).

The average number of vessels in group II glucose induced were significantly more ($p<0.001$) compared to group I sterile water treated. The average number of vessels in group II were 54.00 ± 1.23 compared to sterile water treated group with vessel number 31.00 ± 0.96. The group III glucose induced eggs treated with EAF-PG showed an average number of

vessel of 42.33 ± 1.05 which was significantly less ($p<0.01$) compared to group II eggs (Table.13 and figure. 23)

The blood glucose levels were measured in blood samples taken from CAM vessels. The blood glucose level of group I normal eggs (vehicle treated) were 73.50 ± 1.25mg/dl. The blood samples taken from CAM vessels of group II eggs showed an average blood glucose levels of 304.8 ± 2.85 mg/dl which was significantly high ($p<0.001$) compared to group I vehicle treated eggs. The blood glucose level of group III glucose induced eggs treated with EAF-PG 100 µg showed an average blood glucose glucose level of 117.0 ± 2.63 mg/dl which was significantly less compared to group II glucose induced eggs. There was a significant decrease in blood glucose level between group II and group III eggs (Table.14 and Figure.28)

DISCUSSION

Angiogenesis is the physiologic condition characterized by the growth of new blood vessels originated from preexisting ones. The angiogenic process follows several steps: first of all, a number of angiogenic growth factors activate the receptors present on resident endothelial cells. Once activated, the endothelial cells begin to release specific enzymes called proteases that degrade the basement membrane, finally allowing endothelial cells to leave the original (parental) vessel wall. At this stage, endothelial cells proliferate into the surrounding matrix, taking advantage of adhesion molecules called integrins followed by proliferation of the endothelial cells, vessels elongation, vessels branching, vasodilatation, formation of basement membrane, acquisition of pericyte, and re-modelling (West and Burbridge, 2009). Angiogenesis may represent a pharmacological target for combating diseases characterized by either poor vascularization or hypertrophic vasculature.

Antiangiogenic therapies, in particular, are presently employed to fight cancer and other malignancies (Tremolada et al. 2012**)**.

The angiogenic switch takes place when the positive angiogenic factors such as VEGF outweigh the negative factors such as endostatin (Folkman, 2002). Therefore, the angiogenic switch may result from over production of VEGF. Since angiogenesis is indispensable for tumour growth and metastasis, early in 1971, Folkman proposed that the growth and metastasis of tumor could be reduced by inhibiting angiogenesis (Folkman, 1971). Recently more efforts have been concentrated on synthesis or discovery of non-cytotoxic compounds that have antiangiogenic and anti-neoplastic activity. This treatment approach decreases the side effects that accompany the classical chemotherapeutics drugs.

Concerning the eye, the angiogenic process has to be considered as a pathologic phenomenon. There are actually several conditions leading to the formation of abnormal neovascularization. Age-related macular degeneration is one of the most important diseases characterized by the formation of choroidal new vessel in the macular region finally leading; if untreated, to vision loss. The other major disease characterized by abnormal formation of retinal vessels is diabetic retinopathy, in particular the so-called proliferative stage of this disease (Tremolada et al. 2012**)**. Chronic and sustained hyperglycemia works as a trigger to the early alterations that culminate in vascular dysfunction (Capitao and Soares 2016). Hyperglycemia can cause diabetic micro- (UK Prospective Diabetes Study (UKPDS) Group) and macrovascular complications (Holman et al. 2008) by triggering oxidative stress [Brownlee 2001; Paget et al. 1998), forming advanced glycation end products (AGEs) (Paget et al. 1998; Basta et al. 2002), increasing the flux of glucose to sorbitol through the polyol and hexosamine pathways (Brownlee 2001), activating protein kinase C (PKC) (Ishii et al. 1998), and provoking inflammation (Shanmugam et al. 2004). More importantly, activation

of PKC can induce endothelial cells to release entothelin-1, which is a potent vasoconstrictor that may decrease retinal perfusion, and vascular endothelial growth factors (VEGFs) that may break down the blood-brain barrier and also lead to neovascularisation (Knot 2003).

Since angiogenesis is critical for tumor growth and metastasis, abnormal neovascularization and vascular dysfunction in diabetic retinopathy, anti-angiogenic and vaculoprotective treatments will be a highly promising therapeutic approach. Thus, for over last couple of decades, there has been a robust activity aimed towards the discovery of angiogenesis inhibitors. More than forty anti-angiogenic drugs are being tested in clinical trials all over the world. Many studies have been focused on screening compounds or extracts from natural products for antiangiogenic properties (Aisha et al., 2009; Wang et al., 2004). With the goal of finding a potent antiangiogenic and vasculoprotective drug, an initiative was taken to screen widely known medicinal plant *Punica granatum*. Encouraged from the promising results of previous studies on *Punica granatum* roots, in this study *Punica granatum* roots were evaluated for anti-angiogenic and vaculoprotective property in chorioallantoic membrane model. Since CAM model is used as an effective model for investigating angiogenesis, including anti-angiogenic drugs (Richardson and Singh 2003), CAM model was selected to study the effect of *Punica granatum* roots. Crude ethanol extract of *Punica granatum* root were evaluated for angiogenesis modulatory role in chorioallantoic membrane. Crude ethanol extract of *Punica granatum root* at a concentration of 100 µg showed a potent inhibition on microvascular formation in developing CAM during embryogenesis. Since crude ethanol extract of *Punica granatum* showed a potent inhibition of angiogenesis in CAM assay model, EEPG was subjected to successive fractionation using solvents of increasing polarity (Petroleum ether, chloroform, ethyl acetate, n-butanol, aqueous). The fractions obtained were subjected to evaluation for angiogenesis modulatory

role in chorioallantoic membrane. From the results, it was observed that ethyl acetate fraction at concentration of 100 µg showed a potent in inhibition of angiogenesis in CAM assay. Based on the results obtained from chorioallantoic membrane assay, ethyl acetate fraction was evaluated for vasculoprotective property against glucose induced vascular changes in CAM model. Hyperglycaemia was induced by single intravitellus injection of 5 mg glucose/g whole egg. On day 12 of incubation, blood glucose level was measured in the samples taken from the CAM vessel using Accu-check glucometer and the blood vessels were inspected for any vascular change/abnormalities. A significant increase in blood glucose was noted in glucose induced eggs with microvascular abnormalities including vascular leakage with haemorrhagic spots (microaneurysms), proliferation of new vessels (neovascularization), superficial white lesions were observed. Ethyl acetate fraction at concentration of 100 µg showed a significant protection against glucose induced microvascular abnormalities with a significant decrease in blood glucose level. The proliferation of new vessels (neovascularization) in glucose induced eggs is might be due to vascular occlusion that is responsible for ischemia. Ischemia, via hypoxia-inducible transcription factors (HIF-1 α and -2α), will trigger a coordinated response of angiogenesis by inducing the expression of growth factors, such as the vascular endothelial growth factor (VEGF), the angiopoietins, and the transforming growth factor-β1 (TGF-β1) and their receptors, and by decreasing expression of natural inhibitors (e.g., thrombospondin-1, pigment epithelium-derived factor) (Larger et al. 2004). Due to fragile and having thin wall, these newly formed blood vessels are leaky, which leak the blood on the surface. Also in hyperglycemic there will be increase in diacylglycerol generation (DAG), advanced glycosylation end product (AGE) and free radical generation which activate PKC receptor by DAG-PKC pathway cause capillary degeneration and vascular permeability **(**Brownlee 2001). The significant protection excerted by *Punica granatum* root against glucose induced vascular abnormality in CAM is might be

due to anti-angiogenic and anti-oxidant nature. All parts of the **pomegranate** were reported to contain high levels of ellagitannins. **Pomegranate** root was reported root to contain high levels of ellagitannins. Upon consumption, ellagitannins are hydrolyzed to ellagic acid which is reported to be anti-angiogenic. The root part is also rich in punicalin and punicalagin. Both punicalagin and punicalin can be hydrolyzed to ellagic acid, a natural phenol with high antioxidant activity that can prevent the advanced glycosylation end product (AGE) and free radical generation that cause capillary degeneration and vascular permeability. Since anti-oxidants are well known potent anti-angiogenic (Lobo et al. 2010). The potency of EAF-PG in inhibiting new blood vessel development could be contributed to its significant antioxidant behaviour. One of the potent angiogenic agents is transforming growth factor alpha TGFα; antioxidant agents can inhibit TGFα expression as one aspect of their ability to inhibit angiogenesis (Teo and HO, 2013). It is also suggested that the extract might contain active constituents that repress the expression of VEGF and VEGF like growth factors thereby inhibiting the formation of blood vessels.

SUMMARY AND CONCLUSION

The effect of *punica granatum* roots on angiogenesis and vasoprotection was evaluated by chorioallantoic membrance (CAM) model. Powdered *Punica granatum* roots were extracted with ethanol by hot continuous extraction method. The crude ethanol extract of *Punica granatum* was subjected to preliminary photochemical examination and quantitative analysis. The free radical scavenging activity of extract was evaluated by DPPH radical scavenging and nitric oxide scavenging activity. It was observed that the extract was rich in alkaloids, flavonoids, tannins, saponins, glycoside and possessed a potent free radical scavenging activity.

The extract was screened for angiogenesis modulation in chorioallantoic membranel. In CAM model, a significant decrease in average number of blood vessels was noted in extract treated group compared to vehicle control eggs. The extract was subjected to fractionation using solvents of increasing potency and the fractions were subjected to evaluation of anti-angiogenesis activity in CAM. Among the fractions, the ethyl acetate fraction showed a potent angiogenesis inhibition compared to vehicle control. Based on these results the ethyl acetate fraction was evaluated against glucose induced vascular changed in CAM model. To induce hyperglycemia in developing embryo, a single intravitellus injection of 5 mg glucose/g whole egg was introduced. The treatment groups received ethyl acetate fraction and 5 mg glucose/g whole egg. On day 12 of incubation, the blood glucose levels were measured in blood samples taken from CAM vessels and vascular changes were examined in CAM. Hyperglycaemia with vascular leakage, haemorrhagic spots (microaneurysms), proliferation of new vessels (neovascularization), superficial lesions were spotted in untreated eggs. Ethyl acetate fraction 100 µg showed a significant protection against glucose induced microvascular abnormalities with a significant decrease in blood

glucose level and reduced average number of blood vessels. These findings demonstrate the anti-angiogenic and vasculoprotective effect of *Punica granatum* against hyperglycaemia induced vascular changes in CAM model.

In conclusion, root extract of *Punica granatum* possess a significant angiogenesis inhibition in chorioallantoic membrane assay and vasuloprotective effect in glucose induced vascular change. The possible mechanism of antiangiogenesis and vasoprotective actions are might be due to the presence of active principles that possess a potent antioxidant property of roots, as antioxidants are potent inhibitors of angiogenesis and might be due to the presence of inhibitors that repress the expression of VEGF and VEGF like growth factors thereby inhibiting the formation of new blood vessels and maintaining a vascular stability by inhibiting capillary degeneration and permeability. Thus the isolation of antiangiogenic active principle from *Punica granatum* roots could bring hope to millions of sufferers with cancer and diabetic retinopathy. Further work is in progress to identify the bioactive compounds and delineate the underlying mechanism of antiangiogenesis and vasoprotection.

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